Characterization of the transovarial transmission potential, tissue tropisms and genetic determinants of host specificity of single-host flaviviruses

by

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DEDICATION

I dedicate this dissertation to my family: my mom, dad, sister and brothers. I appreciate your endless love and support.

I also dedicate this dissertation to my mentor, Dr. Bradley Blitvich. This dissertation would not have been accomplished without your guidance, encouragement and support. Thank you so much.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Page

CHAPTER 2

CHAPTER 4

ABSTRACT

Most known flaviviruses, including West Nile virus (WNV), are maintained in natural transmission cycles between hematophagous arthropods and vertebrate hosts; thus, they are dual-host viruses. Other flaviviruses such as Modoc virus (MODV) and Culex flavivirus (CxFV) are single-host viruses because they have host ranges restricted to vertebrates and insects, respectively. Numerous insect-specific flaviviruses (ISFs) including CxFV have been discovered in the last decade and most are widely spread in nature. However, little is known about the mechanism(s) by which ISFs are maintained in nature. In a previous study, CxFV was detected in both female and male mosquitoes collected in the field suggesting that this virus is maintained in nature by vertical transmission. The experiments outlined in chapter 2 were designed to test the hypothesis that efficient transovarial transmission (TOT) of CxFV occurs in the mosquito host. CxFV RNA was detected in 526 of 540 *Culex pipiens* progeny derived from CxFV-infected females and thus, the filial infection rate was 97.4%. Because all positive females produced infected offspring, the TOT prevalence was 100%. These data indicated that extremely efficient TOT of CxFV occurs in mosquitoes in nature. Tissue tropisms of CxFV were also defined. CxFV RNA was detected in all tissues tested: salivary glands, ovaries, testes, head, fat bodies and midguts. Time course experiments demonstrated that CxFV disseminates to the ovaries as early as 4 days post-inoculation. In chapter 3, the host range and genetic diversity of CxFV was investigated. Previously, a high prevalence of CxFV was reported in *Cx. quinquefasciatus* in the Yucatan

Peninsula of Mexico. To determine whether other *Culex* spp. mosquitoes in this region are susceptible to natural CxFV infection, five other *Culex* spp. mosquitoes were tested for evidence of CxFV infection. Two pools of *Cx. interrogator* were positive. The envelope protein genes of these isolates and 16 isolates from *Cx. quinquefasciatus* were sequenced and shown to have ≥99.2% nucleotide identity. These data suggest that there is limited genetic diversity among CxFV isolates in Yucatan Peninsula of Mexico. In chapter 4, studies were performed to increase our knowledge of the genetic elements that condition the differential host ranges of flaviviruses. Although flaviviruses possess a similar genomic organization, they differ in terms of their host specificity; some flaviviruses infect both vertebrates and arthropods whereas others have a vertebrate-specific or arthropod-specific phenotype. The genetic elements that condition these differential host ranges and transmission cycles have not been identified. Therefore, chimeric viruses were constructed by replacing the capsid (C), premembrane (prM) and envelope (E) genes or the prM-E genes of MODV with the corresponding regions of WNV and CxFV. Chimeric virus was recovered in cells transfected with the fusion product containing the prM-E genes of WNV in a MODV backbone. The virus could infect vertebrate but not mosquito cells, indicating that genetic elements outside of the prM-E gene region of MODV condition its vertebrate-specific phenotype. The three other chimeras did not produce detectable virus. Comparative studies between flaviviruses that possess differential host range profiles will help us understand why some flaviviruses can infect only vertebrate or only invertebrate organisms while other

flaviviruses can infect both insect and vertebrate hosts and cause devastating disease in humans and animals.

CHAPTER 1

GENERAL INTRODUCTION

Introduction

Most known members of the genus *Flavivirus* (family *Flaviviridae*) are arthropod-borne viruses which are transmitted horizontally between vertebrate hosts and hematophagous vectors (i.e. mosquitoes and ticks). These dual-host flaviviruses include human and animal pathogens of global concern such as all four serotypes of dengue virus (DENV), yellow fever virus (YFV), Japanese encephalitis virus (JEV) and West Nile virus (WNV) (Gubler et al., 2007). In contrast, other viruses in this genus such as Modoc virus (MODV) have a vertebrate host but no known arthropod vector (NKV) and thus, are assumed to be vertebrate-specific (or single-host) viruses. Another group of flaviviruses contains viruses such as Culex flavivirus (CxFV) which have been isolated from mosquitoes but have no apparent vertebrate host. These viruses are considered to be insect-specific and thus, are also single-host. Single-host viruses are believed to have a higher evolutionary rate than dual-host viruses because they have the potential to adapt to specific-hosts without the need to compromise fitness level in alternate hosts in order to maximize their overall fitness (Major et al., 2009; Novella et al., 1995; Weaver et al., 1999). On the other hand, dual-host viruses are exposed to fitness constraints imposed by disparate biological systems that preclude specific adaption to either host (Ciota and Kramer, 2010; Ciota et al., 2008; Deardorff et al., 2011; Lobo et al., 2009; Vasilakis et al., 2009). Comparative studies between single- and dual-host members of the *Flavivirus*

genus will provide us with more knowledge not only on viral evolution, host specificity, and viral transmissibility and may also provide insight on emerging and re-emerging diseases as well as useful information for creating efficient disease control and prevention strategies (i.e. vaccine development).

Although insect-specific flaviviruses (ISFs) do not appear to infect or cause disease in humans or vertebrate animals, additional research on this group of flaviviruses is still warranted. ISFs have a wide range geographic distribution that overlaps with mosquito-borne flaviviruses, and ISFs infect the primary vectors of pathogenic flaviviruses such as WNV and JEV (Crabtree et al., 2003; Hoshino et al., 2007; Huhtamo et al., 2009; Obara-Nagoya et al., 2013). Several studies have reported co-infection of mosquitoes with mosquito-borne and ISFs in nature (Bolling et al., 2012; Morales-Betoulle et al., 2008; Newman et al., 2011) but the impact that ISFs have on the transmission of pathogenic flaviviruses is still unknown. Co-circulation of ISFs and mosquito-borne flaviviruses in nature leads to questions regarding the interactions between these two groups of flaviviruses in vector populations (Crabtree et al., 2003). Numerous ISFs have been discovered in the last decade and most of them are widely spread in nature, however, we still do not have much information on transmission dynamics of these ISFs. This dissertation contains a study to investigate a mechanism by which a representative ISF (CxFV) is maintained in nature as well as a study to determine the tissue tropisms of this virus in *Culex pipiens* mosquitoes (Chapter 2). In addition, a study to investigate the mosquito host range of CxFV and genetic diversity of this virus isolated from field-collected mosquitoes is included (Chapter 3). Comparative

studies between ISFs and arthropod-borne flaviviruses are important because they will help us understand why some flaviviruses such as WNV can infect and cause devastating disease in humans and vertebrate animals while other flaviviruses such as CxFV do not.

Arthropod-borne flaviviruses can infect both vertebrate and invertebrate organisms while NKVs and ISFs have host ranges restricted to vertebrates and invertebrates respectively. At present, the genetic elements that condition the differential host ranges and transmission cycles of flaviviruses have not been identified. To address this issue, the experiments in chapter 4 were conducted using representative viruses from the vertebrate-specific, insect-specific and arthropod-borne flavivirus groups (MODV, CxFV and WNV, respectively) to construct chimeric viruses and to characterize their *in vitro* host ranges. This study increases our knowledge of the genetic elements that mediate the vastly different host ranges and transmissibilities of these viruses.

Dissertation Organization

This dissertation consists of 5 chapters. The first chapter presents general background and the objectives of each study followed by a literature review which provides general knowledge of the genus *Flavivirus* and information which is related to the topic of the studies. Chapters 2 and 3 include manuscripts in the same format as they appear in their respective peer-reviewed scientific journals. Chapter 4 is a manuscript that has been submitted for publication. The final chapter, chapter 5, provides general conclusions that summarize the outcomes of each study as well as suggestions for future research. All figures and tables appear after the reference section of their respective chapter.

Literature Review

Flavivirus genus

The genus *Flavivirus* (Cook and Holmes, 2006) consists of more than 70 viruses and most of them are arthropod-borne viruses (Ciota et al., 2008) which are transmitted between vertebrate hosts by arthropod vectors such as mosquitoes and ticks. Many flaviviruses are human and animal pathogens of global importance: DENV, YFV, JEV and WNV (Barrett and Higgs, 2007; Gubler, 2006; Gubler et al., 2007; Kramer et al., 2007; Mackenzie et al., 2004). Although there are human vaccines available for YFV, JEV and tick-borne encephalitis virus, there is no specific treatment or effective antiviral therapy for any other flavivirus. According to the WHO, approximately 2.5 billion people around the world are at risk for DENV infection and 50 to 100 million cases occurred each year.

WNV was first isolated from the blood of a woman in Uganda in 1937 (Smithburn, 1940). Historically, the areas endemic for WNV are Africa, Europe, Asia and Australia. However, in 1999, WNV was introduced to the U.S. and rapidly spread across the Western Hemisphere and is now found on all continents except Antarctica (Ciota and Kramer, 2013; Kramer et al., 2007). This outbreak of WNV is the most widespread of arboviruses in the world, and is the biggest encephalitic disease outbreak ever reported in the Western Hemisphere. WNV is maintained in nature in an enzootic transmission cycle between *Culex* species mosquitoes and birds. Humans, horses and other non-avian vertebrate animals usually serve as incidental (or dead-end) hosts because they are unable to develop sufficient viremic titers to infect mosquito vectors

(Blitvich, 2008; Hayes et al., 2005; Kramer et al., 2007). Eighty percent of WNV infections in humans are asymptomatic and 20% result in a mild flu-like illness. Approximately 1% of symptomatic cases develop severe neurological symptoms (Hayes and Gubler, 2006).

Virion morphology and genome structure

Flaviviruses are small spherical enveloped viruses of approximately 50 nm in diameter with an icosahedral nucleocapsid structure that contains multiple copies of the capsid (C) protein. The viral envelope consists of a lipid bilayer which is acquired from the host cell and in which 180 copies of the envelope (E) and membrane (M) proteins are embedded (Lindenbach et al., 2013).

All flaviviruses have a similar genomic structure (Harris et al., 2006; Lindenbach et al., 2013). The genome is composed of a single stranded positive-sense RNA molecule of approximately 11 kb with a type I 5' cap, $m^7GpppAmpN_2$, and a non-polyadenylated 3' end (Lindenbach et al., 2013). The genome contains a long single open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTRs) of ~100 and 400-700 nucleotide, respectively (Markoff, 2003). Translation of the ORF generates a large poly protein that is co- and post-translationally cleaved into three structural proteins designated the capsid (C), premembrane/membrane (prM/M) and envelope (E) proteins, and at least seven non-structural (NS) proteins in the gene order: 5′–C–prM(M)–E–NS1–NS2A–NS2B–NS3–NS4A–NS4B–NS5-3′ (Castle et al., 1986; Castle et al., 1985; Rice et al., 1985). Host signal peptidases cleave viral proteins at the cleavage sites between C/prM, prM/E, E/NS1, and NS4A/NS4B. Virus serine protease

cleaves viral proteins at the cleavage sites between NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5. The enzyme that cuts between NS1 and NS2A remains unknown (Lindenbach et al., 2013). Interestingly, all known ISFs uniquely encode an additional gene: a novel overlapping gene in the NS2A-NS2B region that is the result of a -1 ribosomal frameshift (Firth et al., 2010).

According to phylogenetic analyses (Figure 1) flaviviruses can be categorized into three major groups: arthropod-borne flaviviruses (which can be further divided into mosquito-borne and tick-borne groups), no known arthropod vector (vertebrate-specific) flaviviruses and insect-specific flaviviruses (Cook and Holmes, 2006; Cook et al., 2012; Gould et al., 2003; Lobo et al., 2009). Because insect-specific flaviviruses form the most distant lineage in phylogenies, it has been postulated that they are primitive flaviviruses from which the other members in the genus evolved (Cook and Holmes, 2006).

Flavivirus life cycle

The process of a flavivirus entering the cell begins with interactions between viral envelope glycoprotein (E) and host cellular receptors (Brinton, 2002). Virus particles enter host cells via clathrin-mediated endocytosis (Gollins and Porterfield, 1985; van der Schaar et al., 2008). Flaviviruses can also enter mammalian host cells by directly penetrating the plasma membrane (Hase et al., 1989; Vancini et al., 2013). After the virus particles are internalized into an endosome, low pH triggers a fusion reaction between viral and cellular membrane and the virus nucleocapsid is released into the cytoplasm (Chu and Ng, 2004b; Gollins and Porterfield, 1985; Gollins and Porterfield, 1986). The process of viral genome RNA translation and replication are closely

associated with the rough endoplasmic reticulum (RER). Presumably, the mechanisms that flaviviruses use in the mosquito cell cytoplasm during viral genome translation and replication is similar to that used in mammalian cells, although the kinetics may be slower due to the lower body temperature of mosquitoes (Olson and Blair, 2012). The viral RNA replication complex is composed of several NS proteins including the viral helicase and protease (NS3), viral protease cofactor (NS2B), and RNA-dependent RNA polymerase and methytransferase (NS5) as well as various host factors (Egloff et al., 2002; Falgout et al., 1991; Lindenbach and Rice, 2003; Tan et al., 1996). RNA replication starts with negative-strand RNA synthesis, and the resulting negative-stranded RNA then serves as a template for the production of additional positive-stranded genomic RNAs (Lindenbach and Rice, 2003). Virion assembly appears to occur quickly. Assembly of the C protein and viral genomic RNA is believed to occur by budding into the ER lumen to acquire an envelope (Deubel and Digoutte, 1981; Hase et al., 1987a, b; Ishak et al., 1988; Ko et al., 1979; Leary and Blair, 1980; Mackenzie and Westaway, 2001; Matsumura et al., 1977; Ohyama et al., 1977; Sriurairatna and Bhamarapravati, 1977). Budding at the plasma membrane also has been reported (Hase et al., 1987b; Matsumura et al., 1977; Ohyama et al., 1977; Sriurairatna and Bhamarapravati, 1977) but it is not a major mechanism for virion formation (Lindenbach and Rice, 2003). Emerging virus particles are transported to the cell surface via the host secretory pathways and are released by exocytosis. Maturation of virions occurs as they travel through the host secretory pathway, concurrent with cleavage of prM into pr and M by the Golgi-resident furin or a furin-like enzyme (Stadler et al., 1997). It is believed

that the pr fragment protects the E protein from undergoing an irreversible conformational change as the virions are transported in acidic endosomes in the early exocytosis partway (Guirakhoo et al., 1992; Guirakhoo et al., 1991).

Flaviviruses infect various types of cells and several cell surface proteins have been described as putative receptors. Several studies have been conducted in different host species derived cell lines to investigate host cell receptors that are utilized by flaviviruses (Rodenhuis-Zybert et al., 2010). It has been suggested that multiple receptors could be employed by flaviviruses during cell entry (Smit et al., 2011). In mammalian cells, various molecules have been reported to be involved in flavivirus attachment and entry including: negative charged glycoaminoglycans (heparin sulfate) (Chen et al., 2010; Germi et al., 2002; Hilgard and Stockert, 2000; Kozlovskaya et al., 2010; Lee and Lobigs, 2008; Mandl et al., 2001), C-type lectins such as dendritic cell-specific intracellular adhesion molecule 3-grabbing non integrin (DC-SIGN), DC-SIGN related protein (Davis et al., 2006a; Davis et al., 2006b; Lozach et al., 2005; Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003), a mannose receptor (Miller et al., 2008), $\alpha_v\beta_3$ integrins (Chu and Ng, 2004a; Lee et al., 2006), GRP78 (BiP) (Jindadamrongwech et al., 2004), heat-shock proteins 90 and 70 (Reyes-del Valle et al., 2005), and a 37-kDa/67-kDa laminin receptor (Thepparit and Smith, 2004). In addition, several flaviviruses can enter cells via opsonization by the cells bearing Fc and/or complement receptors (Cardosa et al., 1983; Halstead and O'Rourke, 1977; Schlesinger and Brandriss, 1981). There is limited information regarding the molecules that serve as receptors in mosquito cells. R80 and R67 in C6/36 (*Ae. albopictus*) cells and the midgut

cells of *Ae. aegypti* have been reported to interact with four serotypes of DENV (Mercado-Curiel et al., 2006). A 45-kDa protein has shown to be a part of receptor complex to mediate DENV4 to enter C6/36 cells (Yazi Mendoza et al., 2002).

Insect-specific flaviviruses

Viruses in this group have no apparent vertebrate host and they are assumed to be insect-specific because they do not replicate in mice or any vertebrate cell lines. The first ISF to be discovered was cell fusing agent virus (CFAV) after it was isolated from an *Aedes aegypti* mosquito cell line over 35 years ago (Stollar and Thomas, 1975). Seventeen years later, the complete genome of this virus was sequenced (Cammisa-Parks et al., 1992). Shortly after, CFAV was isolated from field-collected *Ae. aegypti*, *Ae. albopictus* and *Culex* spp. mosquitoes in Puerto Rico (Cook et al., 2006), *Ae. aegypti* and *Ae. albopictus* in Indonesia (Hoshino et al., 2009), and *Ae. aegypti* in Thailand (Kihara et al., 2007) and Mexico (Espinoza-Gómez et al., 2011). The virus causes severe cytopathic effect (CPE) with intensive syncytium formation in C6/36 cells (Crabtree et al., 2003; Stollar and Thomas, 1975). Isolations of CFAV from both male and female mosquito pools collected in Puerto Rico provide evidence for vertical transmission of this virus (Cook et al., 2006).

Kamiti River virus (KRV) was the second insect-specific flavivirus to be discovered. The virus was isolated from *Ae. macintoshi* larvae and pupae in Kenya in 1999 (Crabtree et al., 2003; Sang et al., 2003). KRV produces CPE in C6/36 cells but does not produces syncytium formation unlike C6/36 cells infected with CFAV

(Crabtree et al., 2003). Vertical transmission of KRV was demonstrated in a laboratory study of orally infected *Ae. aegypti* mosquitoes (Lutomiah et al., 2007).

Culex flavivirus (CxFV) was the third ISF to be discovered. CxFV was first isolated from *Cx. pipiens* and *Cx. tritaeniorhynchus* in Japan in 2003, and *Cx. quinquefasciatus* in Indonesia in 2004 (Hoshino et al., 2007). CxFV was isolated from various species of *Culex* mosquitoes including *Cx. pipiens*, *Cx. quinquefasciatus*, *Cx. tritaeniorhynchus*, *Cx.restuans*, *Cx. tarsalis* and *Cx. interrogator* and has since been detected throughout much of the world including the United States (Texas, Iowa, California, Colorado and Illinois), Latin America (Guatemala, Mexico, Trinidad, and Brazil), Africa (Uganda), and Asia (Indonesia, Japan, and China) (Blitvich et al., 2009; Bolling et al., 2011; Cook et al., 2009; Farfan-Ale et al., 2009; Hoshino et al., 2007; Huanyu et al., 2012; Kim et al., 2009; Morales-Betoulle et al., 2008; Newman et al., 2011; Saiyasombat et al., 2010; Tyler et al., 2011). Phylogenies constructed using E gene sequences divided CxFV into two clades. One clade consists of isolates from Latin America, the Caribbean, and Africa and the other clade consists of isolates from North America and Asia (Blitvich et al., 2009; Kim et al., 2009; Saiyasombat et al., 2010). Not all strains of CxFV cause CPE in C6/36 cells. The first isolate from Japan causes mild CPE in only after 4 passages (Hoshino et al., 2007). Some strains of CxFV do not cause CPE in C6/36 cells including the isolates from Guatemala (Morales-Betoulle et al., 2008), Mexico (Farfan-Ale et al., 2009) and Uganda (Cook et al., 2009). One out of 7 strains from Texas (Kim et al., 2009) and all strains from Iowa (Blitvich et al., 2009)

produce obvious CPE and syncytial formation in C6/36 cells. The CxFV isolate recently reported in China causes obvious CPE within 3-4 days (Huanyu et al., 2012).

The detection of CxFV from both male and female mosquitoes suggests vertical transmission of the virus (Bolling et al., 2011; Farfan-Ale et al., 2009; Hoshino et al., 2007) and recent data indicate that transovarial transmission is a major mechanism for CxFV to be maintained in nature (Saiyasombat et al., 2011). Horizontal transmission from male to female mosquitoes is probably another mechanism for CxFV maintenance in nature but it presumably plays a minor role. Bolling et al. (2012) reported evidence of venereal transmission in *Cx. pipiens* from male to female and interestingly from female to male mosquitoes. This is the only study to provide evidence of sexual transmission of a flavivirus from female to male mosquitoes, thus, additional experiments are needed to support this finding. In addition, there was no evidence of horizontal transmission occurring in the larval mosquitoes when CxFV infected- and uninfected larvae were reared together (Bolling et al., 2012).

Other ISFs have been discovered in recent years including Quang Binh virus (QBV) isolated from *Cx. tritaeniorhychus* in Vietnam (Crabtree et al., 2009), Aedes flavivirus (AeFV) in Japan (Hoshino et al., 2009), Nakiwoko virus in Uganda (Cook et al., 2009), Lammi virus in Finland (Huhtamo et al., 2009), Nounané virus in Côte d'Ivoire, Africa (Junglen et al., 2009), Calbertado virus in California , Colorado and Alberta (Bolling et al., 2011; Tyler et al., 2011), Culex theileri flavivirus in Portugal (Parreira et al., 2012), and Palm Creek virus (PCV) in Australia (Hobson-Peters et al., 2013). Currently, more than 20 ISFs have been discovered (Haddow et al., 2013).

Although ISFs have been isolated mostly from mosquitoes, ISF-like RNA was identified by RT-PCR and nucleotide sequencing in *Phlebotomine* sandflies (Moureau et al., 2010; Sanchez-Seco et al., 2009). Additionally ISF-like DNA sequences, named "cell silent agents" (CSA), have been found integrated into mosquito genomes (Crochu et al., 2004; Roiz et al., 2009; Vázquez et al., 2012). This indicates a close relationship between insect flaviviruses and their mosquito hosts in nature. CSA is most closely related to AeFV (Crochu et al., 2004; Hoshino et al., 2007).

Based on available phylogenetic data, ISFs can be divided into two clades; one clade contains *Aedes*-associated viruses such as CFAV, AeFV and KRV, and the other clade contains *Culex*-associated viruses such as CxFV and QBV. NAKV also belongs to the second clade even though it was isolated from *Mansonia* spp. mosquitoes. Nevertheless, in using E gene sequences to construct phylogenies, CFAV was grouped with QBV instead of KRV and this occurs with both laboratory and field isolates of CFAV (Cook et al., 2012; Hoshino et al., 2007).

Flavivirus co-infections in mosquito hosts

Several ISFs were detected at a high prevalence in mosquitoes in the area in which they were discovered (Farfan-Ale et al., 2009; Hobson-Peters et al., 2013). CxFV has been isolated from *Cx. tritaeniorhynchus* which is primary vector of JEV in Asia (Obara-Nagoya et al., 2013). CxFV has been detected in various *Culex* species mosquitoes which can potentially transmit WNV (Hoshino et al., 2007). More importantly, ISFs have wide geographic distribution that overlaps with arthropod-borne flaviviruses of public health concern. Although ISFs do not have a direct effect by

causing disease in humans and vertebrate animals, they may have indirect effect on humans and animal health. For example, they may increase or decrease the transmissibility of pathogenic flavivirus by co-infected arthropod vectors.

Persistent infection with one virus can interfere with subsequent infection by a closely-related virus through a process called superinfection exclusion (or homologous interference) (Tscherne et al., 2007). Superinfection exclusion has been observed during infections by a broad range of viruses and has been reported to occur in both vertebrate and invertebrate cells (Barbanti-Brodano et al., 1970; Condreay and Brown, 1986). Zou et al. (2009) demonstrated that WNV replicons can prevent superinfection of WNV and other flaviviruses but not non-flaviviruses in baby hamster kidney cells and the exclusion process occurred during RNA synthesis. Superinfection of arboviruses in mosquitoes and mosquito cell lines has been investigated previously. For example, C6/36 cells persistently infected with Sindbis virus were resistant to superinfection with other strains of Sindbis virus or other alphaviruses (Karpf et al., 1997). Persistent infection of *Ae. albopictus* and *Ae. dorsalis* cell lines with St. Louis encephalitis virus (SLEV) causes the cells to become refractory to infection with other strains of SLEV, but not JEV or YFV (Randolph and Hardy, 1988). *In vivo* studies in *Ae. aegypti* mosquitoes, which are natural vectors for both DENV and YFV, showed that *Ae. aegypti* infected with DENV were less likely to become infected with and subsequently transmit YFV compared to DENV uninfected mosquitoes (Sabin, 1952). *Cx. quinquefasciatus* mosquitoes sequentially infected with either WNV or SLEV have lower infection and

dissemination rates for the second virus compared to single-virus infected controls (Pesko and Mores, 2009).

There is limited data to date regarding interactions between ISFs and arboviruses during *in vitro* and *in vivo* studies and the effect of the co-infection on vector competence for arboviruses. Bolling et al. (2012) reported significantly lower WNV titers in C6/36 cells that had been previously infected with CxFV compared to cells infected with WNV alone. Suppression of WNV replication occurred at earlier time points (between 84 and 156 hr post infection). A similar study by Kent et al. (2010) also showed lower titers of WNV in C6/36 cells infected with CxFV compared to cells infected with WNV alone but these differences were not statistically significant. Recently, Hobson-Peters et al. (2013) demonstrated that prior infection of mosquito cells with an ISF known as PCV suppresses the replication of WNV and Murray Valley encephalitis virus. For CxFV, which is *Culex* mosquitoes associated, it would be better to perform future studies in *Culex* cells line instead of C6/36 cells which are derived from *Ae. albopictus* cells. Additionally, the lack of innate immune response for virus infection in mosquito cell lines such as C6/36 cells (Brackney et al., 2010; Scott et al., 2010), could lead to the different results between *in vitro* and *in vivo* experiment.

Interestingly, significantly higher WNV transmission rates occurred with *Cx. quinquefasciatus* mosquitoes that had been co-infected with WNV and the CxFV Izabal strain from Guatemala compared to mosquitoes infected with only WNV (Kent et al., 2010). WNV-positive pools of field collected *Cx. pipiens* mosquitoes from Illinois were four times more likely to be infected with CxFV than WNV-negative pools from the

same area, and 40% of individual WNV-infected mosquito pools were also CxFV positive (Newman et al., 2011). In contrast, Bolling et al. (2012) showed that WNV replication and dissemination were suppressed in early time points in *Cx. pipiens* mosquitoes persistently infected with CxFV. The dissemination rate of WNV was significantly higher in *Cx. pipiens* mosquitoes from a CxFV uninfected colony compared to *Cx. pipiens* mosquitoes from colony that is persistently infected with CxFV. However, there is no significant difference in transmission rates between these two colonies and so the impact on vector competence for WNV in mosquitoes co-infected with CxFV is still unclear (Bolling et al., 2012).

The above data provide evidence that ISFs may have an impact on the transmission of pathogenic flaviviruses in nature. Due to the limited amount of data to date and the variations of results that have been reported, further studies are still needed to clarify interactions between ISFs and arthropod-borne flaviviruses in arthropod hosts in nature. Nevertheless, according to the broad range of genetic diversity within ISFs and within arthropod-borne flaviviruses, variable outcomes may be observed due to variable strains of viruses and mosquitoes species.

No Known Arthropod Vector flaviviruses

Flaviviruses that are assigned to this group have no known apparent arthropod vector and do not replicate in mosquito or tick cell lines (Singh, 1972; Varelas-Wesley and Calisher, 1982). NKV flaviviruses have been isolated exclusively from rodents and bats. Phylogenetic studies performed using NS5 gene sequences showed a cluster of NKVs that is closer to the root of the tree than tick-borne and mosquito-borne

flaviviruses. These findings indicate that NKVs are primordial flaviviruses or that NKVs and arthropod-borne flaviviruses evolved from the primordial flavivirus and then arthropod-borne viruses separated into tick-borne and mosquito-borne clusters. The NS5 phylogram also divided NKV viruses into 3 clades; clade I and II contain viruses mostly isolated from rodents and clade III contains viruses isolated from bats (Kuno et al., 1998).

The first NKV flavivirus to be discovered was Rio Bravo virus which was isolated from bats in Texas in 1956 (Burns and Farinacci, 1956). This was followed by the discovery of Modoc virus (MODV) from rodents in 1958 (Johnson, 1967). Other NKVs that have also been isolated from rodents include Apoi virus from Japan, Cowbone Ridge virus from Florida, Jutiapa virus from Guatemala and Salvieja and San Perlita viruses from Texas. Other NKV flaviviruses isolated from bats include Bukalasa bat virus from Uganda, Cary Island virus from Malaysia, Dakar bat virus from Senegal, Montana myotis leukoencephalitis virus from North America, and Phnom Penh bat virus from Cambodia (Gubler et al., 2007). Information on NKV flaviviruses is limited although in the past few years these viruses have received more attention as demonstrated by several recent phylogenetic studies (Billoir et al., 2000; Kuno et al., 1998) as well as recent studies investigating the potential of these viruses to serve as models for finding treatments for pathogenic flavivirus infections (Leyssen et al., 2001).

MODV is a murine flavivirus that was first isolated from the mammary gland tissue of a white footed deer mouse (*Peromyscus maniculatus*) in Modoc county, California in 1958 (Johnson, 1967). This was followed by the isolation of MODV from a

boy with aseptic meningitis in California (Davis and Hardy, 1973), however, the detection of neutralizing antibodies in humans and animals in Alberta indicated that infection by this virus without disease can occur in nature (Zarnke and Yuill, 1985). Later, MODV was isolated from deer mice in Oregon, Montana, Colorado, and Alberta, Canada. It has been suggested that MODV is maintained in nature by horizontal transmission via direct close contact between host populations (Fairbrother and Yuill, 1987). MODV can cause persistent infection in deer mice and hamsters with chronic viruria (Adams et al., 2013; Davis and Hardy, 1974; Davis et al., 1974; Johnson, 1970). Regardless of an effective antibody response, infected hamsters chronically shed virus in the urine for up to 4 months after infection (Adams et al., 2013). Similar results of persistent renal infection and continuously shed virus in the urine for a long period of time also found in experimentally infected-golden hamster with WNV (Tesh et al., 2005). Within the same group of NKV, Rio Bravo virus also causes persistent infection in bats. This virus accumulates in salivary gland and shed in the saliva. It is up to almost 2 years that the virus can be detected in salivary glands (Constantine and Woodall, 1964). MODV cause severe encephalitis in SCID mice and hamsters. SCID mice and hamsters infected with MODV by intracerebral, intranasal, or intraperitoneal inoculations have been developed as a model for encephalitic flavivirus infection because the infected animals showed histopathological signs that are very similar to signs presented in humans by other encephalitic flaviviruses (Leyssen et al., 2001).

References

Adams, A.P., Travassos da Rosa, A.P.A., Nunes, M.R., Xiao, S.-Y., Tesh, R.B., 2013, Pathogenesis of Modoc Virus (*Flaviviridae; Flavivirus*) in Persistently Infected Hamsters. The American Journal of Tropical Medicine and Hygiene 88, 455-460.

Barbanti-Brodano, G., Swetly, P., Koprowski, H., 1970, Superinfection of simian virus 40-transformed permissive cells with simian virus 40. J Virol 6, 644-651.

Barrett, A.D., Higgs, S., 2007, Yellow fever: a disease that has yet to be conquered. Annu Rev Entomol 52, 209-229.

Billoir, F., de Chesse, R., Tolou, H., de Micco, P., Gould, E.A., de Lamballerie, X., 2000, Phylogeny of the genus flavivirus using complete coding sequences of arthropod-borne viruses and viruses with no known vector. J Gen Virol 81, 781-790.

Blitvich, B.J., 2008, Transmission dynamics and changing epidemiology of West Nile virus. Anim Health Res Rev 9, 71-86.

Blitvich, B.J., Lin, M., Dorman, K.S., Soto, V., Hovav, E., Tucker, B.J., Staley, M., Platt, K.B., Bartholomay, L.C., 2009, Genomic sequence and phylogenetic analysis of Culex flavivirus, an insect-specific flavivirus, isolated from *Culex pipiens* (Diptera: Culicidae) in Iowa. J Med Entomol 46, 934-941.

Bolling, B.G., Eisen, L., Moore, C.G., Blair, C.D., 2011, Insect-Specific Flaviviruses from *Culex* Mosquitoes in Colorado, with Evidence of Vertical Transmission. The American Journal of Tropical Medicine and Hygiene 85, 169-177.

Bolling, B.G., Olea-Popelka, F.J., Eisen, L., Moore, C.G., Blair, C.D., 2012, Transmission dynamics of an insect-specific flavivirus in a naturally infected *Culex pipiens* laboratory colony and effects of co-infection on vector competence for West Nile virus. Virology 427, 90-97.

Brackney, D.E., Scott, J.C., Sagawa, F., Woodward, J.E., Miller, N.A., Schilkey, F.D., Mudge, J., Wilusz, J., Olson, K.E., Blair, C.D., Ebel, G.D., 2010, C6/36 *Aedes albopictus* Cells Have a Dysfunctional Antiviral RNA Interference Response. PLoS Negl Trop Dis 4, e856.

Brinton, M.A., 2002, The Molecular Biology of West Nile Virus: A New Invader of the Western Hemisphere. Annual Review of Microbiology 56, 371-402.

Burns, K.F., Farinacci, C.J., 1956, Virus of bats antigenically related to St. Louis encephalitis. Science 123, 227.

Cammisa-Parks, H., Cisar, L.A., Kane, A., Stollar, V., 1992, The complete nucleotide sequence of cell fusing agent (CFA): Homology between the nonstructural proteins encoded by CFA and the nonstructural proteins encoded by arthropod-borne flaviviruses. Virology 189, 511-524.

Cardosa, M.J., Porterfield, J.S., Gordon, S., 1983, Complement receptor mediates enhanced flavivirus replication in macrophages. The Journal of Experimental Medicine 158, 258-263.

Castle, E., Leidner, U., Nowak, T., Wengler, G., Wengler, G., 1986, Primary structure of the West Nile flavivirus genome region coding for all nonstructural proteins. Virology 149, 10-26.

Castle, E., Nowak, T., Leidner, U., Wengler, G., Wengler, G., 1985, Sequence analysis of the viral core protein and the membrane-associated proteins V1 and NV2 of the flavivirus west nile virus and of the genome sequence for these proteins. Virology 145, 227-236.

Chen, H.-L., Her, S.-Y., Huang, K.-C., Cheng, H.-T., Wu, C.-W., Wu, S.-C., Cheng, J.-W., 2010, Identification of a heparin binding peptide from the Japanese encephalitis virus envelope protein. Peptide Science 94, 331-338.

Chu, J.J.-h., Ng, M.-L., 2004a, Interaction of West Nile Virus with αvβ3 Integrin Mediates Virus Entry into Cells. Journal of Biological Chemistry 279, 54533-54541.

Chu, J.J.H., Ng, M.L., 2004b, Infectious Entry of West Nile Virus Occurs through a Clathrin-Mediated Endocytic Pathway. Journal of Virology 78, 10543-10555.

Ciota, A., Kramer, L., 2013, Vector-Virus Interactions and Transmission Dynamics of West Nile Virus. Viruses 5, 3021-3047.

Ciota, A.T., Kramer, L.D., 2010, Insights into Arbovirus Evolution and Adaptation from Experimental Studies. Viruses 2, 2594-2617.

Ciota, A.T., Lovelace, A.O., Jia, Y., Davis, L.J., Young, D.S., Kramer, L.D., 2008, Characterization of mosquito-adapted West Nile virus. Journal of General Virology 89, 1633-1642.

Condreay, L.D., Brown, D.T., 1986, Exclusion of superinfecting homologous virus by Sindbis virus-infected *Aedes albopictus* (mosquito) cells. Journal of Virology 58, 81-86.

Constantine, D.G., Woodall, D.F., 1964, Latent Infection of Rio Bravo Virus in Salivary Glands of Bats. Public Health Reports (1896-1970) 79, 1033-1039.

Cook, S., Bennett, S.N., Holmes, E.C., De Chesse, R., Moureau, G., de Lamballerie, X., 2006, Isolation of a new strain of the flavivirus cell fusing agent virus in a natural mosquito population from Puerto Rico. Journal of General Virology 87, 735-748.

Cook, S., Holmes, E.C., 2006, A multigene analysis of the phylogenetic relationships among the flaviviruses (Family: *Flaviviridae*) and the evolution of vector transmission. Archives of Virology 151, 309-325.

Cook, S., Moureau, G., Harbach, R., Mukwaya, L., Goodger, K., Ssenfuka, F., Gould, E., Holmes, E.C., de Lamballerie, X., 2009, Isolation of a new species of flavivirus and a novel strain of Culex flavivirus (*Flaviviridae*), from a natural mosquito population in Uganda. J Gen Virol.

Cook, S., Moureau, G., Kitchen, A., Gould, E.A., de Lamballerie, X., Holmes, E.C., Harbach, R.E., 2012, Molecular evolution of the insect-specific flaviviruses. Journal of General Virology 93, 223-234.

Crabtree, M.B., Nga, P.T., Miller, B.R., 2009, Isolation and characterization of a new mosquito flavivirus, Quang Binh virus, from Vietnam. Arch Virol.

Crabtree, M.B., Sang, R.C., Stollar, V., Dunster, L.M., Miller, B.R., 2003, Genetic and phenotypic characterization of the newly described insect flavivirus, Kamiti River virus. Archives of Virology 148, 1095-1118.

Crochu, S., Cook, S., Attoui, H., Charrel, R.N., De Chesse, R., Belhouchet, M., Lemasson, J.-J., de Micco, P., de Lamballerie, X., 2004, Sequences of flavivirus-related RNA viruses persist in DNA form integrated in the genome of *Aedes* spp. mosquitoes. Journal of General Virology 85, 1971-1980.

Davis, C.W., Mattei, L.M., Nguyen, H.-Y., Ansarah-Sobrinho, C., Doms, R.W., Pierson, T.C., 2006a, The Location of Asparagine-linked Glycans on West Nile Virions Controls Their Interactions with CD209 (Dendritic Cell-specific ICAM-3 Grabbing Nonintegrin). Journal of Biological Chemistry 281, 37183-37194.

Davis, C.W., Nguyen, H.-Y., Hanna, S.L., Sánchez, M.D., Doms, R.W., Pierson, T.C., 2006b, West Nile Virus Discriminates between DC-SIGN and DC-SIGNR for Cellular Attachment and Infection. Journal of Virology 80, 1290-1301.

Davis, J.W., Hardy, J.L., 1973, In Vitro Studies with Modoc Virus in Vero Cells: Plaque Assay and Kinetics of Growth, Neutralization, and Thermal Inactivation. Applied Microbiology 26, 344-348.

Davis, J.W., Hardy, J.L., 1974, Characterization of Persistent Modoc Viral Infections in Syrian Hamsters. Infection and Immunity 10, 328-334.

Davis, J.W., Hardy, J.L., Reeves, W.C., 1974, Modoc Viral Infections in the Deer Mouse Peromyscus maniculatus. Infection and Immunity 10, 1362-1369.

Deardorff, E.R., Fitzpatrick, K.A., Jerzak, G.V.S., Shi, P.-Y., Kramer, L.D., Ebel, G.D., 2011, West Nile Virus Experimental Evolution *in vivo* and the Trade-off Hypothesis. PLoS Pathog 7, e1002335.

Deubel, V., Digoutte, J.P., 1981, Morphogenesis of Yellow Fever Virus in Aedes aegypti Cultured Cells: I. Isolation of Different Cellular Clones and the Study of Their Susceptibility to Infection with the Virus. The American Journal of Tropical Medicine and Hygiene 30, 1060-1070.

Egloff, M.P., Benarroch, D., Selisko, B., Romette, J.L., Canard, B., 2002, An RNA cap (nucleoside-2′-O-)-methyltransferase in the flavivirus RNA polymerase NS5: crystal structure and functional characterization. The EMBO Journal 21, 2757-2768.

Espinoza-Gómez, F., López-Lemus, A., Rodriguez-Sanchez, I., Martinez-Fierro, M., Newton-Sánchez, O., Chávez-Flores, E., Delgado-Enciso, I., 2011, Detection of sequences from a potentially novel strain of cell fusing agent virus in Mexican *Stegomyia (Aedes) aegypti* mosquitoes. Archives of Virology 156, 1263-1267.

Fairbrother, A., Yuill, T.M., 1987, Experimental infection and horizontal transmission of Modoc virus in deer mice (*Peromyscus maniculatus*). Journal of Wildlife Diseases 23, 179-185.

Falgout, B., Pethel, M., Zhang, Y.M., Lai, C.J., 1991, Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. Journal of Virology 65, 2467-2475.

Farfan-Ale, J.A., Lorono-Pino, M.A., Garcia-Rejon, J.E., Hovav, E., Powers, A.M., Lin, M., Dorman, K.S., Platt, K.B., Bartholomay, L.C., Soto, V., Beaty, B.J., Lanciotti, R.S., Blitvich, B.J., 2009, Detection of RNA from a Novel West Nile-like virus and high prevalence of an insect-specific flavivirus in mosquitoes in the Yucatan Peninsula of Mexico. Am J Trop Med Hyg 80, 85-95.

Farfan-Ale, J.A., Lorono-Pino, M.A., Garcia-Rejon, J.E., Soto, V., Lin, M., Staley, M., Dorman, K.S., Bartholomay, L.C., Hovav, E., Blitvich, B.J., 2010, Detection of flaviviruses and orthobunyaviruses in mosquitoes in the Yucatan Peninsula of Mexico in 2008. Vector Borne Zoonotic Dis 10, 777-783.

Firth, A.E., Blitvich, B.J., Wills, N.M., Miller, C.L., Atkins, J.F., 2010, Evidence for ribosomal frameshifting and a novel overlapping gene in the genomes of insect-specific flaviviruses. Virology 399, 153-166.

Germi, R., Crance, J.-M., Garin, D., Guimet, J., Lortat-Jacob, H., Ruigrok, R.W.H., Zarski, J.-P., Drouet, E., 2002, Heparan Sulfate-Mediated Binding of Infectious Dengue Virus Type 2 and Yellow Fever Virus. Virology 292, 162-168.

Gollins, S.W., Porterfield, J.S., 1985, Flavivirus Infection Enhancement in Macrophages: an Electron Microscopic Study of Viral Cellular Entry. Journal of General Virology 66, 1969-1982.

Gollins, S.W., Porterfield, J.S., 1986, The Uncoating and Infectivity of the Flavivirus West Nile on Interaction with Cells: Effects of pH and Ammonium Chloride. Journal of General Virology 67, 1941-1950.

Gould, E.A., de Lamballerie, X., Zanotto, P.M.d.A., Holmes, E.C., 2003, Origins, evolution, and vector□host coadaptations within the Genus *Flavivirus*, In: Advances in Virus Research. Academic Press, pp. 277-314.

Gubler, D.J., 2006, Dengue/dengue haemorrhagic fever: history and current status. Novartis Found Symp 277, 3-16; discussion 16-22, 71-13, 251-253.

Gubler, D.J., Kuno, G., Markoff, L., 2007, Flaviviruses. In Fields Virology. Fifth Edition. Editors: Knipe, D.M., Howley, P.M. pp. 1153-1252. Lippincott Williams and Wilkins. Philadelphia, PA.

Guirakhoo, F., Bolin, R.A., Roehrig, J.T., 1992, The Murray Valley encephalitis virus prM protein confers acid resistance to virus particles and alters the expression of epitopes within the R2 domain of E glycoprotein. Virology 191, 921-931.

Guirakhoo, F., Heinz, F.X., Mandl, C.W., Holzmann, H., Kunz, C., 1991, Fusion activity of flaviviruses: comparison of mature and immature (prM-containing) tick-borne encephalitis virions. The Journal of general virology 72 (Pt 6), 1323-1329.

Haddow, A.D., Guzman, H., Popov, V.L., Wood, T.G., Widen, S.G., Haddow, A.D., Tesh, R.B., Weaver, S.C., 2013, First isolation of Aedes flavivirus in the Western Hemisphere and evidence of vertical transmission in the mosquito *Aedes (Stegomyia) albopictus* (Diptera: Culicidae). Virology 440, 134-139.

Halstead, S., O'Rourke, E., 1977, Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. The Journal of Experimental Medicine 146, 201-217.

Harris, E., Holden, K.L., Edgil, D., Polacek, C., Clyde, K., 2006, Molecular biology of flaviviruses. Novartis Found Symp 277, 23-39; discussion 40, 71-23, 251-253.

Hase, T., Summers, P.L., Eckels, K.H., 1989, Flavivirus entry into cultured mosquito cells and human peripheral blood monocytes. Archives of Virology 104, 129-143.

Hase, T., Summers, P.L., Eckels, K.H., Baze, W.B., 1987a, An electron and immunoelectron microscopic study of dengue-2 virus infection of cultured mosquito cells: Maturation events. Archives of Virology 92, 273-291.

Hase, T., Summers, P.L., Eckels, K.H., Baze, W.B., 1987b, Maturation process of Japanese encephalitis virus in cultured mosquito cells in vitro and mouse brain cells in vivo. Archives of Virology 96, 135-151.

Hayes, E.B., Gubler, D.J., 2006, West Nile Virus: Epidemiology and Clinical Features of an Emerging Epidemic in the United States*. Annual Review of Medicine 57, 181-194.

Hayes, E.B., Komar, N., Nasci, R.S., Montgomery, S.P., O'Leary, D.R., Campbell, G.L., 2005, Epidemiology and transmission dynamics of West Nile virus disease. Emerg Infect Dis 11, 1167-1173.

Hilgard, P., Stockert, R., 2000, Heparan Sulfate Proteoglycans Initiate Dengue Virus Infection of Hepatocytes. Hepatology 32, 1069-1077.

Hobson-Peters, J., Yam, A.W.Y., Lu, J.W.F., Setoh, Y.X., May, F.J., Kurucz, N., Walsh, S., Prow, N.A., Davis, S.S., Weir, R., Melville, L., Hunt, N., Webb, R.I., Blitvich, B.J., Whelan, P., Hall, R.A., 2013, A New Insect-Specific Flavivirus from Northern Australia Suppresses Replication of West Nile Virus and Murray Valley Encephalitis Virus in Co-infected Mosquito Cells. PLoS ONE 8, e56534.

Hoshino, K., Isawa, H., Tsuda, Y., Kazuhiko, Y., Toshinori, S., Yuda, M., Takasaki, T., Kobayashi, M., Sawabe, K., 2007, Genetic characterization of a new insect flavivirus isolated from *Culex pipiens* mosquito in Japan. Virology 359, 405 - 414.

Hoshino, K., Isawa, H., Tsuda, Y., Sawabe, K., Kobayashi, M., 2009, Isolation and characterization of a new insect flavivirus from Aedes albopictus and *Aedes flavopictus* mosquitoes in Japan. Virology 391, 119-129.

Huanyu, W., Haiyan, W., Shihong, F., Guifang, L., Hong, L., Xiaoyan, G., Lizhi, S., Rayner, S., Aiqiang, X., Guodong, L., 2012, Isolation and identification of a distinct strain of Culex Flavivirus from mosquitoes collected in Mainland China. Virology Journal 9, 1-8.

Huhtamo, E., Putkuri, N., Kurkela, S., Manni, T., Vaheri, A., Vapalahti, O., Uzcátegui, N.Y., 2009, Characterization of a Novel Flavivirus from Mosquitoes in Northern

Europe That Is Related to Mosquito-Borne Flaviviruses of the Tropics. Journal of Virology 83, 9532-9540.

Ishak, R., Tovey, D.G., Howard, C.R., 1988, Morphogenesis of Yellow Fever Virus 17D in Infected Cell Cultures. Journal of General Virology 69, 325-335.

Jindadamrongwech, S., Thepparit, C., Smith, D.R., 2004, Identification of GRP 78 (BiP) as a liver cell expressed receptor element for dengue virus serotype 2. Arch Virol 149, 915-927.

Johnson, H.N., 1967, Ecological implications of antigenically related mammalian viruses for which arthropod vectors are unknown and avian associated soft tick viruses. Jpn J Med Sci Biol 20 Suppl, 160-166.

Johnson, H.N., 1970, Long-Term Persistence of Modoc Virus in Hamster-Kidney Cells: In vivo and in vitro Demonstration. The American Journal of Tropical Medicine and Hygiene 19, 537-539.

Junglen, S., Kopp, A., Kurth, A., Pauli, G., Ellerbrok, H., Leendertz, F.H., 2009, A New Flavivirus and a New Vector: Characterization of a Novel Flavivirus Isolated from *Uranotaenia* Mosquitoes from a Tropical Rain Forest. Journal of Virology 83, 4462-4468.

Karpf, A.R., Lenches, E., Strauss, E.G., Strauss, J.H., Brown, D.T., 1997, Superinfection exclusion of alphaviruses in three mosquito cell lines persistently infected with Sindbis virus. J Virol 71, 7119-7123.

Kent, R.J., Crabtree, M.B., Miller, B.R., 2010, Transmission of West Nile Virus by *Culex quinquefasciatus* Say Infected with Culex Flavivirus Izabal. PLoS Negl Trop Dis 4, e671.

Kihara, Y., Satho, T., Eshita, Y., Sakai, K., Kotaki, A., Takasaki, T., Rongsriyam, Y., Komalamisra, N., Srisawat, R., Lapcharoen, P., Sumroiphon, S., Iwanaga, S., Ushijima, H., Endoh, D., Miyata, T., Sakata, A., Kashige, N., Miake, F., Fukushi, S., Saijo, M., Kurane, I., Morikawa, S., Mizutani, T., 2007, Rapid determination of viral RNA sequences in mosquitoes collected in the field. Journal of Virological Methods 146, 372-374.

Kim, D.Y., Guzman, H., Bueno, R., Jr., Dennett, J.A., Auguste, A.J., Carrington, C.V., Popov, V.L., Weaver, S.C., Beasley, D.W., Tesh, R.B., 2009, Characterization of Culex Flavivirus (Flaviviridae) strains isolated from mosquitoes in the United States and Trinidad. Virology 386, 154-159.

Ko, K.K., Igarashi, A., Fukai, K., 1979, Electron microscopic observations on *Aedes albopictus* cells infected with dengue viruses. Archives of Virology 62, 41-52.

Kozlovskaya, L.I., Osolodkin, D.I., Shevtsova, A.S., Romanova, L.I., Rogova, Y.V., Dzhivanian, T.I., Lyapustin, V.N., Pivanova, G.P., Gmyl, A.P., Palyulin, V.A., Karganova, G.G., 2010, GAG-binding variants of tick-borne encephalitis virus. Virology 398, 262-272.

Kramer, L.D., Styer, L.M., Ebel, G.D., 2007, A Global Perspective on the Epidemiology of West Nile Virus. Annu Rev Entomol.

Kuno, G., Chang, G.J., Tsuchiya, K.R., Karabatsos, N., Cropp, C.B., 1998, Phylogeny of the genus *Flavivirus*. J Virol 72, 73-83.

Leary, K., Blair, C.D., 1980, Sequential events in the morphogenesis of Japanese encephalitis virus. Journal of Ultrastructure Research 72, 123-129.

Lee, E., Lobigs, M., 2008, E Protein Domain III Determinants of Yellow Fever Virus 17D Vaccine Strain Enhance Binding to Glycosaminoglycans, Impede Virus Spread, and Attenuate Virulence. Journal of Virology 82, 6024-6033.

Lee, J.W.-M., Chu, J.J.-H., Ng, M.-L., 2006, Quantifying the Specific Binding between West Nile Virus Envelope Domain III Protein and the Cellular Receptor αVβ3 Integrin. Journal of Biological Chemistry 281, 1352-1360.

Leyssen, P., Van Lommel, A., Drosten, C., Schmitz, H., De Clercq, E., Neyts, J., 2001, A Novel Model for the Study of the Therapy of Flavivirus Infections Using the Modoc Virus. Virology 279, 27-37.

Lindenbach, B.D., Murray, C.L., Thiel, H.-J., Rice, C.M., 2013, *Flaviviridae*. In Fields Virology, Sixth Edition. Editors: Knipe, D.M. Howley, P.M. pp. 712-746. Lippincott Williams and Wilkins. Philadelphia, PA.

Lindenbach, B.D., Rice, C.M., 2003, Molecular biology of flaviviruses. Adv Virus Res 59, 23-61.

Lobo, F.P., Mota, B.E.F., Pena, S.D.J., Azevedo, V., Macedo, A.M., Tauch, A., Machado, C.R., Franco, G.R., 2009, Virus-Host Coevolution: Common Patterns of Nucleotide Motif Usage in *Flaviviridae* and Their Hosts. PLoS ONE 4, e6282.

Lozach, P.-Y., Burleigh, L., Staropoli, I., Navarro-Sanchez, E., Harriague, J., Virelizier, J.-L., Rey, F.A., Desprès, P., Arenzana-Seisdedos, F., Amara, A., 2005, Dendritic Cell-specific Intercellular Adhesion Molecule 3-grabbing Non-integrin (DC-SIGN)-mediated Enhancement of Dengue Virus Infection Is Independent of DC-SIGN Internalization Signals. Journal of Biological Chemistry 280, 23698-23708.

Lutomiah, J.J.L., Mwandawiro, C., Magambo, J., Sang, R.C., 2007, Infection and Vertical Transmission of Kamiti River Virus in Laboratory Bred *Aedes aegypti* Mosquitoes. Journal of Insect Science 7, 1-7.

Mackenzie, J.M., Westaway, E.G., 2001, Assembly and Maturation of the Flavivirus Kunjin Virus Appear to Occur in the Rough Endoplasmic Reticulum and along the Secretory Pathway, Respectively. Journal of Virology 75, 10787-10799.

Mackenzie, J.S., Gubler, D.J., Petersen, L.R., 2004, Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat Med 10, S98-109.

Major, L., Linn, M.L., Slade, R.W., Schroder, W.A., Hyatt, A.D., Gardner, J., Cowley, J., Suhrbier, A., 2009, Ticks Associated with Macquarie Island Penguins Carry Arboviruses from Four Genera. PLoS ONE 4, e4375.

Mandl, C.W., Kroschewski, H., Allison, S.L., Kofler, R., Holzmann, H., Meixner, T., Heinz, F.X., 2001, Adaptation of Tick-Borne Encephalitis Virus to BHK-21 Cells Results in the Formation of Multiple Heparan Sulfate Binding Sites in the Envelope Protein and Attenuation In Vivo. Journal of Virology 75, 5627-5637.

Markoff, L., 2003, 5′- and 3′-noncoding regions in flavivirus RNA, In: Advances in Virus Research. Academic Press, pp. 177-228.

Matsumura, T., Shiraki, K., Sashikata, T., Hotta, S., 1977, Morphogenesis of Dengue-1 Virus in Cultures of a Human Leukemic Leukocyte Line (J-111). Microbiology and Immunology 21, 329-334.

Mercado-Curiel, R., Esquinca-Aviles, H., Tovar, R., Diaz-Badillo, A., Camacho-Nuez, M., Munoz, M., 2006, The four serotypes of dengue recognize the same putative receptors in *Aedes aegypti* midgut and *Ae. albopictus* cells. BMC Microbiology 6, 85.

Miller, J.L., deWet, B.J.M., Martinez-Pomares, L., Radcliffe, C.M., Dwek, R.A., Rudd, P.M., Gordon, S., 2008, The Mannose Receptor Mediates Dengue Virus Infection of Macrophages. PLoS Pathog 4, e17.

Morales-Betoulle, M.E., Monzon Pineda, M.L., Sosa, S.M., Panella, N., Lopez, M.R., Cordon-Rosales, C., Komar, N., Powers, A., Johnson, B.W., 2008, Culex flavivirus isolates from mosquitoes in Guatemala. J Med Entomol 45, 1187-1190.

Moureau, G., Ninove, L., Izri, A., Cook, S., Lamballerie, X.D., Charrel, R.N., 2010, Flavivirus RNA in Phlebotomine Sandflies. Vec Borne Zoon Dis 10, 195-197.

Navarro-Sanchez, E., Altmeyer, R., Amara, A., Schwartz, O., Fieschi, F., Virelizier, J.L., Arenzana-Seisdedos, F., Desprès, P., 2003, Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses. EMBO reports 4, 723-728.

Newman, C.M., Cerutti, F., Anderson, T.K., Hamer, G.L., Walker, E.D., Kitron, U.D., Ruiz, M.O., Brawn, J.D., Goldberg, T.L., 2011, Culex flavivirus and West Nile virus mosquito coinfection and positive ecological association in Chicago, United States. Vec Borne Zoon Dis 11, 1099-1105.

Novella, I.S., Clarke, D.K., Quer, J., Duarte, E.A., Lee, C.H., Weaver, S.C., Elena, S.F., Moya, A., Domingo, E., Holland, J.J., 1995, Extreme fitness differences in mammalian and insect hosts after continuous replication of vesicular stomatitis virus in sandfly cells. Journal of Virology 69, 6805-6809.

Obara-Nagoya, M., Yamauchi, T., Watanabe, M., Hasegawa, S., Iwai-Itamochi, M., Horimoto, E., Takizawa, T., Takashima, I., Kariwa, H., 2013, Ecological and Genetic Analyses of the Complete Genomes of Culex Flavivirus Strains Isolated From *Culex tritaeniorhynchus* and *Culex pipiens* (Diptera: Culicidae) Group Mosquitoes. Journal of Medical Entomology 50, 300-309.

Ohyama, A., Ito, T., Tanimura, E., Huang, S.-C., Hsue, J.-Y., Furu, Y., 1977, Electron Microscopic Observation of the Budding Maturation of Group B Arboviruses. Microbiology and Immunology 21, 535-538.

Olson, K.E., Blair, C.D., 2012, Flavivirus-Vector interactions. In Molecular Virology and Control of Flaviviruses. Editor: Shi, P. pp.297-334. Caister Academic Press.

Parreira, R., Cook, S., Lopes, Â., de Matos, A.P., de Almeida, A.P.G., Piedade, J., Esteves, A., 2012, Genetic characterization of an insect-specific flavivirus isolated from *Culex theileri* mosquitoes collected in southern Portugal. Virus Research 167, 152-161.

Pesko, K., Mores, C.N., 2009, Effect of Sequential Exposure on Infection and Dissemination Rates for West Nile and St. Louis Encephalitis Viruses in *Culex quinquefasciatus*. Vector-Borne and Zoonotic Diseases 9, 281-286.

Randolph, V.B., Hardy, J.L., 1988, Establishment and characterization of St Louis encephalitis virus persistent infections in *Aedes* and *Culex* mosquito cell lines. J Gen Virol 69 (Pt 9), 2189-2198.

Reyes-del Valle, J., Chávez-Salinas, S., Medina, F., del Angel, R.M., 2005, Heat Shock Protein 90 and Heat Shock Protein 70 Are Components of Dengue Virus Receptor Complex in Human Cells. Journal of Virology 79, 4557-4567.

Rice, C.M., Lenches, E.M., Eddy, S.R., Shin, S.J., Sheets, R.L., Strauss, J.H., 1985, Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. Science 229, 726+.

Rodenhuis-Zybert, I., Wilschut, J., Smit, J., 2010, Dengue virus life cycle: viral and host factors modulating infectivity. Cell. Mol. Life Sci. 67, 2773-2786.

Roiz, D., Vazquez, A., Seco, M., Tenorio, A., Rizzoli, A., 2009, Detection of novel insect flavivirus sequences integrated in *Aedes albopictus* (Diptera: Culicidae) in Northern Italy. Virology Journal 6, 93.

Sabin, A.B., 1952, Research on Dengue during World War II. The American Journal of Tropical Medicine and Hygiene 1, 30-50.

Saiyasombat, R., Bolling, B.G., Brault, A.C., Bartholomay, L.C., Blitvich, B.J., 2011, Evidence of Efficient Transovarial Transmission of Culex Flavivirus by *Culex pipiens* (Diptera: Culicidae). Journal of Medical Entomology 48, 1031-1038.

Saiyasombat, R., Dorman, K.S., Garcia-Rejon, J.E., Loroño-Pino, M.A., Farfan-Ale, J.A., Blitvich B.J, 2010, Host range and genetic diversity of Culex flavivirus in the Yucatan Peninsula of Mexico. Arch Virol 155, 983-986.

Sanchez-Seco, M., Vazquez, A., Collao, X., Hernandez, L., Aranda, C., Ruiz, S., Tenorio, A., 2009, Surveillance of arboviruses in mosquito wetlands: Detection of new Flavi- and Phleboviruses. Vector Borne Zoonotic Dis.

Sang, R., Gichogo, A., Gachoya, J., Dunster, M., Ofula, V., Hunt, A., Crabtree, M., Miller, B., Dunster, M., 2003, Isolation of a new flavivirus related to Cell-fusing agent virus (CFAV) from field collected flood water *Aedes* mosquitoes sampled from a Dambo in central Kenya. Arch Virol 148, 1085 - 1093.

Schlesinger, J.J., Brandriss, M.W., 1981, Antibody-mediated infection of macrophages and macrophage-like cell lines with 17D-yellow fever virus. Journal of Medical Virology 8, 103-117.

Scott, J.C., Brackney, D.E., Campbell, C.L., Bondu-Hawkins, V., Hjelle, B., Ebel, G.D., Olson, K.E., Blair, C.D., 2010, Comparison of Dengue Virus Type 2-Specific Small RNAs from RNA Interference-Competent and –Incompetent Mosquito Cells. PLoS Negl Trop Dis 4, e848.

Singh, K.R.P., 1972, Growth of Arboviruses in Arthropod Tissue Culture, In: Kenneth M. Smith, M.A.L., Frederik, B.B. (Eds.) Advances in Virus Research. Academic Press, pp. 187-206.

Smit, J., Moesker, B., Rodenhuis-Zybert, I., Wilschut, J., 2011, Flavivirus Cell Entry and Membrane Fusion. Viruses 3, 160-171.

Smithburn, K.C., Hughes, T.P., Burke, A.W., Paul, J.H., 1940, A neurotropic virus isolated from the blood of a native of Uganda. Am J Trop Med Hyg 20, 471-492.

Sriurairatna, S., Bhamarapravati, N., 1977, Replication of dengue-2 virus in *Aedes albopictus* mosquitoes. An electron microscopic study. The American Journal of Tropical Medicine and Hygiene 26, 1199-1205.

Stadler, K., Allison, S.L., Schalich, J., Heinz, F.X., 1997, Proteolytic activation of tick-borne encephalitis virus by furin. Journal of Virology 71, 8475-8481.

Stollar, V., Thomas, V.L., 1975, An agent in the *Aedes aegypti* cell line (Peleg) which causes fusion of *Aedes albopictus* cells. Virology 64, 367-377.

Tan, B.-H., Fu, J., Sugrue, R.J., Yap, E.-H., Chan, Y.-C., Tan, Y.H., 1996, Recombinant Dengue Type 1 Virus NS5 Protein Expressed in *Escherichia coli* Exhibits RNA-Dependent RNA Polymerase Activity. Virology 216, 317-325.

Tassaneetrithep, B., Burgess, T.H., Granelli-Piperno, A., Trumpfheller, C., Finke, J., Sun, W., Eller, M.A., Pattanapanyasat, K., Sarasombath, S., Birx, D.L., Steinman, R.M., Schlesinger, S., Marovich, M.A., 2003, DC-SIGN (CD209) Mediates Dengue Virus Infection of Human Dendritic Cells. The Journal of Experimental Medicine 197, 823-829.

Tesh, R.B., Siirin, M., Guzman, H., Travassos da Rosa, A.P.A., Wu, X., Duan, T., Lei, H., Nunes, M.R., Xiao, S.-Y., 2005, Persistent West Nile Virus Infection in the Golden Hamster: Studies on Its Mechanism and Possible Implications for Other Flavivirus Infections. Journal of Infectious Diseases 192, 287-295.

Thepparit, C., Smith, D.R., 2004, Serotype-Specific Entry of Dengue Virus into Liver Cells: Identification of the 37-Kilodalton/67-Kilodalton High-Affinity Laminin Receptor as a Dengue Virus Serotype 1 Receptor. Journal of Virology 78, 12647-12656.

Tscherne, D.M., Evans, M.J., von Hahn, T., Jones, C.T., Stamataki, Z., McKeating, J.A., Lindenbach, B.D., Rice, C.M., 2007, Superinfection Exclusion in Cells Infected with Hepatitis C Virus. Journal of Virology 81, 3693-3703.

Tyler, S., Bolling, B.G., Blair, C.D., Brault, A.C., Pabbaraju, K., Armijos, M.V., Clark, D.C., Calisher, C.H., Drebot, M.A., 2011, Distribution and Phylogenetic Comparisons of a Novel Mosquito Flavivirus Sequence Present in *Culex tarsalis* Mosquitoes from Western Canada with Viruses Isolated in California and Colorado. The American Journal of Tropical Medicine and Hygiene 85, 162-168.

van der Schaar, H.M., Rust, M.J., Chen, C., van der Ende-Metselaar, H., Wilschut, J., Zhuang, X., Smit, J.M., 2008, Dissecting the Cell Entry Pathway of Dengue Virus by Single-Particle Tracking in Living Cells. PLoS Pathog 4, e1000244.

Vancini, R., Kramer, L.D., Ribeiro, M., Hernandez, R., Brown, D., 2013, Flavivirus infection from mosquitoes in vitro reveals cell entry at the plasma membrane. Virology 435, 406-414.

Varelas-Wesley, I., Calisher, C.H., 1982, Antigenic Relationships of Flaviviruses with Undetermined Arthropod-Borne Status. The American Journal of Tropical Medicine and Hygiene 31, 1273-1284.

Vasilakis, N., Deardorff, E.R., Kenney, J.L., Rossi, S.L., Hanley, K.A., Weaver, S.C., 2009, Mosquitoes Put the Brake on Arbovirus Evolution: Experimental Evolution Reveals Slower Mutation Accumulation in Mosquito Than Vertebrate Cells. PLoS Pathog 5, e1000467.

Vázquez, A., Sánchez-Seco, M., Palacios, G., Molero, F., Reyes, N., Ruiz, S., Aranda, C., Marqués, E., Escosa, R., Moreno, J., Figuerola, J., Tenorio, A., 2012, Novel Flaviviruses Detected in Different Species of Mosquitoes in Spain. Vector Borne and Zoonotic Dis 12, 223-229.

Weaver, S.C., Brault, A.C., Kang, W., Holland, J.J., 1999, Genetic and Fitness Changes Accompanying Adaptation of an Arbovirus to Vertebrate and Invertebrate Cells. Journal of Virology 73, 4316-4326.

Yazi Mendoza, M., Salas-Benito, J.S., Lanz-Mendoza, H., Hernández-Martínez, S., del Angel, R.M., 2002, A putative receptor for dengue virus in mosquito tissues: localization of a 45-kDa glycoprotein. The American Journal of Tropical Medicine and Hygiene 67, 76-84.

Zarnke, R.L., Yuill, T.M., 1985, Modoc-like virus isolated from wild deer mice (*Peromyscus maniculatus*) in Alberta. Journal of Wildlife Diseases 21, 94-99.

Zou, G., Zhang, B., Lim, P.-Y., Yuan, Z., Bernard, K.A., Shi, P.-Y., 2009, Exclusion of West Nile Virus Superinfection through RNA Replication. Journal of Virology 83, 11765-11776.

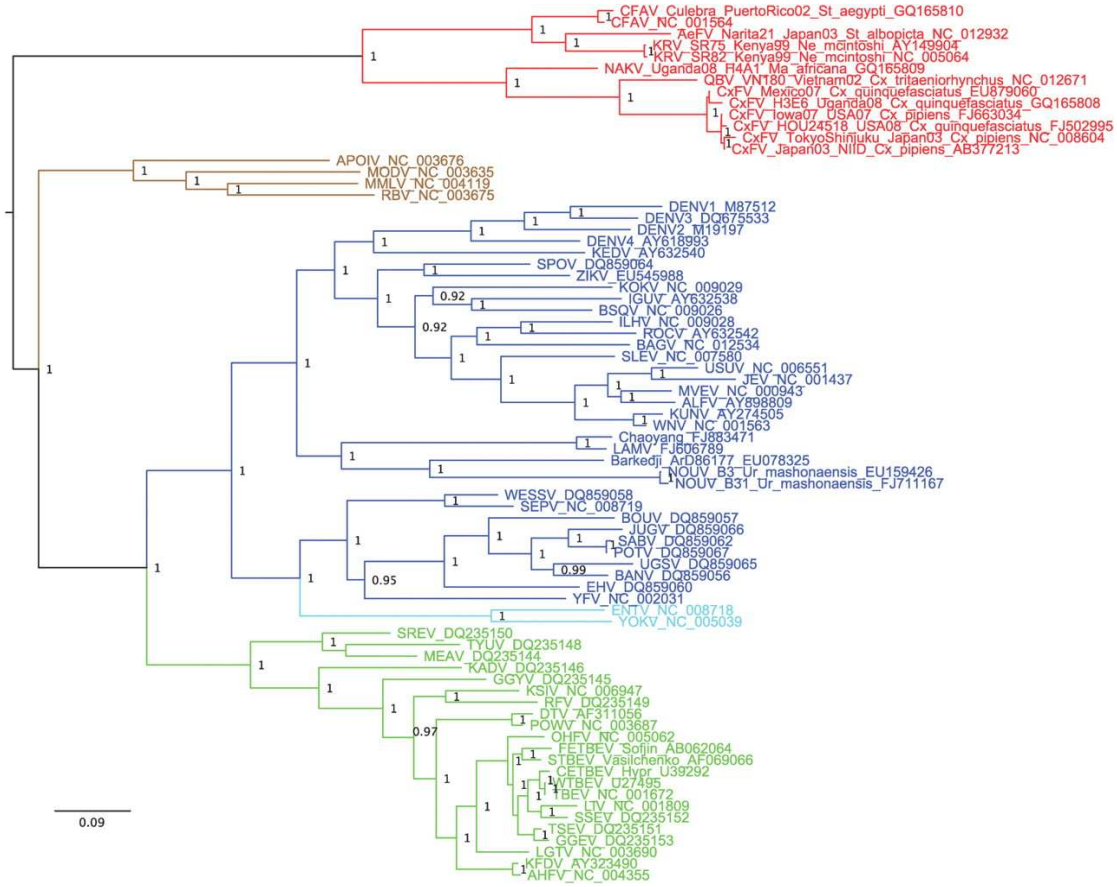

Figure 1 NS5 phylogram shows the genetic relationship among flaviviruses. "*Red, 'insect-specific' flaviviruses; brown, NKV flaviviruses; blue, mosquito-borne flaviviruses; light blue, secondary loss flaviviruses; green, tick-borne flaviviruses.*" (Cook et al., 2012)

CHAPTER 2

EVIDENCE OF EFFICIENT TRANSOVARIAL TRANSMISSION OF CULEX FLAVIVIRUS BY *CULEX PIPIENS* (DIPTERA: CULICIDAE)

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Abstract

This study determined the transovarial transmission (TOT) potential and tissue tropisms of Culex flavivirus (CxFV), an insect-specific flavivirus, in *Culex pipiens* (L.). Several hundred mosquito egg rafts were collected in the field, transferred to the insectaries, reared to the fourth larval instar, and identified using morphological characteristics. *Cx. pipiens* were reared to adults, allowed to oviposit in individual containers and tested for CxFV RNA by reverse transcription-polymerase chain reaction (RT-PCR) and nucleotide sequencing. Eighteen CxFV RNA-positive females were identified from 26 females that oviposited viable egg rafts. Thirty F_1 adults from each positive female were individually tested by RT-PCR for CxFV RNA. Viral RNA was

detected in 526 of 540 progeny and thus, the filial infection rate was 97.4%. Because all 18 positive females produced infected offspring, the TOT prevalence was 100%. These data indicated that efficient TOT of CxFV occurs in nature. To define the tissue tropisms of CxFV, different tissues (salivary glands, ovaries, testes, head, fat bodies and midguts) were removed from the remainder of the F_1 and tested by RT-PCR for CxFV RNA. Viral RNA was detected in all tissues. Additionally, uninfected laboratory-colonized *Cx. pipiens* were infected with CxFV by needle inoculation, and ovaries were collected at 4, 6, 8 and 12 days post-inoculation and tested for CxFV RNA by RT-PCR. Viral RNA was detected at all time points demonstrating that CxFV infects the ovaries as early as 4 days post-inoculation. Surprisingly, however, we were unable to demonstrate transovarial transmission despite the presence of viral RNA in the ovaries. Nevertheless, the experiments performed with field-infected *Cx. pipiens* demonstrate that TOT is an efficient mechanism by which CxFV is maintained in mosquitoes in nature.

Keywords: Flavivirus, Culex flavivirus, *Culex pipiens*, transovarial transmission, tissue tropisms

Introduction

The majority of viruses in the genus *Flavivirus* are transmitted horizontally between vertebrate hosts and hematophagous arthropods such as mosquitoes and ticks (ICTV 2005). Viruses in this group include dengue virus (DENV), yellow fever virus (YFV), Japanese encephalitis virus (JEV) and West Nile virus (WNV), all of which are human pathogens of global importance. Other viruses in this genus are considered to be

vertebrate-specific, because they have a vertebrate host, but no known arthropod vector. Finally, another group of flaviviruses has been isolated strictly from Diptera (mosquitoes and sandflies), has no apparent vertebrate host, and therefore are considered to be insect-specific (Hoshino et al. 2007, Moureau et al. 2009, Sanchez-Seco et al. 2009). Nine insect-specific flaviviruses have been isolated from mosquitoes: Culex flavivirus (CxFV) (Hoshino et al. 2007), cell fusing agent virus (Stollar and Thomas 1975, Cook et al. 2006, Kihara et al. 2007), Kamiti River virus (KRV) (Crabtree et al. 2003, Sang et al. 2003), Quang Binh virus (Crabtree et al. 2009), Aedes flavivirus (Hoshino et al. 2009), Nakiwogo virus (Cook et al. 2009), Lammi virus (Huhtamo et al. 2009), Nounane virus (Junglen et al. 2009) and Calbertado virus (Tyler et al., inpress).

CxFV has a wide geographic distribution, having been isolated from *Culex.* spp. mosquitoes in Asia (Hoshino et al. 2007), Guatemala (Morales-Betoulle et al. 2008), Mexico (Farfan-Ale et al. 2009, Farfan-Ale et al. 2010, Saiyasombat et al. 2010), Trinidad (Kim et al. 2009), the United States (Blitvich et al. 2009, Kim et al. 2009) and Uganda (Cook et al. 2009). In Mexico, CxFV was detected in similar proportions of male and female *Cx. quinquefasciatus*; the CxFV minimal infection rate, expressed as the number of positive mosquito pools per 1,000 mosquitoes tested were 7.2 and 8.3, respectively (Farfan-Ale et al. 2010). These data indicate that CxFV is maintained in nature by vertical transmission, consistent with its vertebrate replication-incompetent phenotype. The isolation of KRV from immature *Aedes macintoshi* (Marks) provides further evidence that vertical transmission of insect-specific flaviviruses occurs in mosquitoes in nature (Sang et al. 2003).

One mechanism of vertical transmission is transovarial transmission (TOT), defined as the process by which progeny of infected females are directly infected in the egg stage within the ovary before release and subsequent insemination. Transovum transmission, in contrast, entails the infection of the egg as it moves down the oviduct. Vertical transmission is believed to be inefficient in mosquitoes infected with arthropod-borne flaviviruses because of low direct infection rates of the ovarian tissue and the requirement for transovum infection during a limited window of time. Alternatively, bunyaviruses with high vertical infection efficiencies frequently exhibit ovariole or follicle infection and undergo TOT. In a classic study, Tesh (1980) compared the vertical infection rates of WNV and DENV to that of a bunyavirus, San Angelo virus, in *Aedes albopictus* (Skuse). Progeny infection rates exceeded 13% for San Angelo virus, but were always <1% for WNV and DENV, even following selection. TOT is also inefficient in mosquitoes infected with yellow fever virus, Japanese encephalitis virus, St. Louis encephalitis virus and Murray Valley encephalitis virus (Rosen et al. 1978, Aitken et al. 1979, Beaty et al. 1980, Kay and Carley 1980, Francy et al. 1981, Hardy et al. 1984). Mosquitoes are very permissive to flavivirus replication and virus antigen is abundant in the ovarian sheath and oviducts, but not in ovarioles or follicles of infected vectors (Rosen 1988, Turell 1988). Clearly, although rarely, flaviviruses are vertically transmitted, and there are occasional reports of isolation of DENV and other flaviviruses from a small proportion of field-collected larvae and male adult mosquitoes. This is frequently called TOT but is a misnomer. Mosquito eggs typically become infected with flaviviruses during insemination (transovum infection) as the egg is moving through the

heavily infected common oviduct (Rosen 1988). During this time the micropyle is open and sperm and fluids can enter the egg for fertilization. Resulting filial transovum infection (FI) rates are very low (<1%) (Aitken et al. 1979, Beaty et al. 1980) especially as compared to the >80% FI rates associated with TOT of La Crosse virus (*Bunyaviridae*) in *Aedes triseriatus* (Say) (Beaty and Bishop 1988, Woodring et al. 1998, Hughes et al. 2006).

As a result of the paucity of data on the mechanism(s) by which insect-specific flaviviruses are maintained in nature, the current study investigated the ability of CxFV to be transovarially transmitted by *Cx. pipiens*. Because CxFV has been detected in similar proportions of female and male mosquitoes in the field (Farfan-Ale et al. 2010) and lacks the capacity to replicate in vertebrates (Hoshino et al. 2007), we tested the hypothesis that efficient TOT of CxFV occurs in the mosquito host.

Materials and Methods

Field-Collected Mosquitoes. Mosquito egg rafts were collected at study sites in three counties (Polk, Roosevelt and Story) in the state of Iowa from September through October 2009 and from July through October 2010. Collections were made using gravid traps containing hay infusion (Lee and Rowley 2000). Mosquitoes were transported to the insectaries at Iowa State University (ISU), reared to the fourth larval stage and identified using morphological characteristics. *Cx. pipiens* were retained; all other species were discarded. Larvae and pupae were reared in polypropylene plastic trays containing tap water supplemented with a slurry of Tetramin. Adult mosquitoes were

maintained on a 10% sucrose solution at 27°C and 80% RH with a light-dark photocycle of 16:8 h.

Laboratory-Colonized Mosquitoes. *Cx. pipiens* (ISU strain) were originally collected as egg rafts at various collection sites in Iowa in 2002 and have been maintained continuously in the insectaries at ISU using the conditions described above. Mosquitoes from this colony are periodically tested for the presence of flavivirus RNA by reverse transcription-polymerase chain reaction (RT-PCR) and continually test negative.

Virus and Titers. CxFV (strain CxFV-Iowa07) was isolated from a pool of *Cx. pipiens* collected in Iowa in 2007 (Blitvich et al. 2009). Because CxFV does not plaque or cause extensive cytopathic effect in mosquito cell culture (Hoshino et al. 2007, Blitvich et al. 2009), the titer of the virus stock was measured by quantitative RT-PCR (qRT-PCR). To correlate qRT-PCR RNA copy determinations with infectivity, 6-well plates of confluent *Ae. albopictus* (C6/36) cells were inoculated with a 10-fold dilution series of CxFV and incubated at 28°C for 9 days. Cells and supernatants were harvested, after which total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and subjected to qRT-PCR as described below. Reed-Muench calculations were employed to estimate infectious units (infectious dose 50%) and to determine specific infectivity of the input virus. The qRT-PCR assay was performed using primers specific to a 207 nucleotide region of the CxFV envelope gene (CxFV-E-Forward, 5'- TGA ATT GCT CGC TGA TTG TC-3' and CxFV-E-Reverse, 5'- TTA TAC CCC TCT CCG CAA TG-3'). Amplification standards were prepared from RNA transcripts produced from a

plasmid generated to contain the first 2,567 nucleotides of the CxFV genome downstream of a T7 RNA polymerase promoter. *In vitro* transcriptions were performed using an AmpliScribe T7 transcription kit (Epicentre Biotechnologies, Madison, WI). Viral RNA was DNase-treated, extracted using TRIzol reagent, and quantified using a spectrophotometer. RNA transcripts were diluted to 10^{10} copies/µl, and 10-fold serial dilutions were used to construct standard curves. Viral RNA was quantified using the Quantitect SYBR Green One-Step RT-PCR kit (Qiagen, Valencia, CA) on a Bio-Rad iCycler iQ5 real-time PCR detection system (BioRad, Hercules, CA). Reactions were performed in duplicate and consisted of 10 µl of Quantitect SYBR Green RT-PCR Master Mix, 0.2 µl Quantitect RT Mix, 1 µl each of forward and reverse primers (10 µM), 10.8 µl of nuclease-free water, and 50 ng of template RNA. Non-template samples and RNA from uninfected C6/36 cells were included as controls. The thermal profile consisted of reverse transcription at 50°C for 30 min, reverse transcriptase inactivation/denaturation at 95°C for 15 min, and 40 cycles of PCR at 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Dissociation analysis was conducted to detect nonspecific amplicons and primer dimers. To avoid the incorporation of nonspecific fluorescence in quantitative measurements, the temperatures at which fluorescence detection was acquired were adjusted to quantify specific products only. Fluorescence profiles from the standard curves were used to directly estimate initial RNA copy numbers of viral genomes in the samples.

TOT Experiments with Field-Collected Mosquitoes. Field-collected *Cx. pipiens* were reared to adults, placed in a single cage for 7 days to facilitate mating, and

then allowed to feed on a quail (*Colinus virginianus*) (Institutional Animal Care and Use Committee Protocol 12-2-5400-Z) to initiate egg development. Before feeding on the quail, mosquitoes were starved by replacing the sucrose solution with water at 24-h pre-blood meal and by removing the water at 6-h pre-blood meal. Engorged females were transferred to individual cartons containing hay infusion water for oviposition. The resulting egg rafts were transferred to individual polypropylene plastic trays containing tap water supplemented with Tetramin. F_1 progeny were removed at the pupal stage and placed into cartons. All F_0 that produced viable eggs were individually tested by RT-PCR and nucleotide sequencing using CxFV-specific primers and a 3730x1 DNA sequencer (Applied Biosystems, Foster City, CA). Thirty F_1 adult progeny (15 females and 15 males) from each CxFV RNA-positive F_0 female were collected on the day of emergence and stored at -80^{o}C prior to being individually tested for CxFV RNA by RT-PCR. The remaining progeny were collected at 8-10 days post-emergence and used for the tissue tropism experiments.

TOT Experiments with Laboratory-Colonized Mosquitoes. Adult female *Cx. pipiens* (4 to 5 days post-emergence) were cold-anesthetized and injected with CxFV by intrathoracic inoculation into the cervical membrane using a fine needle. Each mosquito received an estimated 1.6 x 10^5 50% tissue culture infective dose of CxFV as determined by qRT-PCR. Inoculated females were placed in a cage with uninfected adult males at a ratio of 1:2 to facilitate mating. Mosquitoes were starved as outlined above and, at 8 days post-inoculation, allowed to feed on a quail. Engorged females were transferred to individual oviposition cartons. Egg rafts were transferred to individual cartons, and

mosquitoes were reared to adults as described above. All F_0 females that produced viable eggs were individually tested for CxFV RNA by RT-PCR to confirm that they were infected with CxFV. F_1 progeny were collected on the day of emergence and stored at -80^{o}C until tested by RT-PCR using CxFV-specific primers.

RT-PCR. Total RNA was isolated from whole mosquitoes (either individually or in groups of five) and from mosquito organs (salivary glands, ovaries, testes, head, fat bodies and midguts) previouly ground in TRIzol reagent (Invitrogen) using a mortar and pestle on ice, as described by the manufacturer. Numbers of organs pooled and used in each reaction, denoted in parentheses, are as follows: salivary glands (100), ovaries (100), testes (90), head (5), fat bodies (40) and midguts (90). Total RNA was amplified by RT-PCR using CxFV-specific primers (CxFV-NS5-Forward, 5'-TTG ACT CCA ACG CCT C-3' and CxFV-NS5-Reverse, 5'-ACC TTG AGT TCG AAG CG -3') that target a 446-nucleotide region of the CxFV NS5 gene. Actin-specific primers were included as positive RT-PCR and normalization controls (Staley et al. 2010). Complementary DNAs were generated using Superscript III reverse transcriptase (Invitrogen), and PCRs were performed using *Taq* polymerase (Invitrogen). RT-PCR products were examined by 1% agarose gel electrophoresis and visualized with ethidium bromide staining.

Results

To determine whether CxFV is transovarially transmitted by mosquitoes in the field, several hundred mosquito egg rafts were collected at study sites in Iowa, transferred to the insectaries, reared to the fourth larval stage and identified using

morphological characteristics. *Cx. pipiens* were reared to adults, allowed to mate and offered a blood meal to facilitate egg development. A total of 162 female *Cx. pipiens* produced egg rafts, of which 26 (16%) hatched. Female mosquitoes that produced viable eggs were individually tested for CxFV RNA by RT-PCR and nucleotide sequencing. Eighteen CxFV RNA-positive mosquitoes were identified. BLAST analysis of the resulting sequences revealed that all had ≥99% nucleotide identity to the homologous region of CxFV-Iowa07, the prototype CxFV strain from Iowa (Blitvich et al. 2009).

Thirty F_1 adults (15 females and 15 males) from each CxFV RNA-positive F_0 were collected on the day of emergence and individually assayed by RT-PCR using CxFV-specific primers. Actin-specific primers were included as positive controls. CxFV RNA was detected in 526 of 540 progeny, and thus, the overall estimated FI rate (defined as the percentage of infected F_1 produced from the CxFV RNA-positive females) was 97.4% (Table 1). All 18 CxFV RNA-positive females produced infected progeny and therefore the estimated TOT rate (defined as the percentage of CxFV RNA-positive females that transmitted virus to at least one of their progeny) was 100%. There was no significant difference in the overall proportion of CxFV RNA-positive female and male F_1 (98.5 and 96.3%, respectively; $P = 0.1042$, χ^2 test). There was, however, a significant (albeit modest) difference in the proportion of infected offspring produced by each CxFV-infected F_0 female, with values ranging from 86.7% to 100% ($P = 0.047$, df = 17, χ^2 test).

One additional F_0 female yielded a faint band of the expected size when tested by RT-PCR for the presence of CxFV RNA. Thirty F_1 from this mosquito were then tested

by RT-PCR using CxFV-specific primers. Of these, 9 mosquitoes yielded a strong band of the correct size, 14 yielded a faint positive band and 7 were negative. As a result of the ambiguous nature of these data, they were not included in Table 1 or used to calculate the overall TOT and FI rates. Had these data been included, they would have had a negligible affect on our findings; the overall FI rate would have been 96.3% and the TOT rate would have remained at 100%.

To define the tissue tropism of CxFV, select tissues (salivary glands, ovaries, testes, head, fat bodies and midguts) were removed from the remainder of the F_1 progeny produced from the field-collected *Cx. pipiens* and tested for CxFV RNA by RT-PCR. Actin-specific primers were included as positive controls. CxFV and actin RNA were detected in all tissues (Figure 1). CxFV and/or actin RNA were not detected in the fat bodies and heads when >3µg of total RNA was used in the reverse transcription reactions (data not shown) but were detected when lower quantities of total RNA were used. These data suggest that fat bodies and heads contain dose-dependent inhibitory factors for the enzymatic activity of reverse transcriptase and/or *Taq* polymerase.

To further investigate the TOT potential of CxFV, laboratory-colonized adult female *Cx. pipiens* were infected with CxFV by needle inoculation and transferred to a cage with uninfected adult males. Eight days later, mosquitoes were offered a blood meal, and engorged females were transferred to individual oviposition cartons. RT-PCR analysis confirmed that all 30 F_0 females that produced viable egg rafts were positive for CxFV RNA. A total of 950 F_1 progeny from the CxFV RNA-positive F_0 mosquitoes were tested either individually or in pools of five by RT-PCR using CxFV and actin-

specific primers. All F_1 progeny were negative for CxFV RNA, but positive for actin RNA (data not shown). To establish whether these mosquitoes were refractory to TOT because the virus was unable to disseminate to their ovaries, a second cohort of laboratory-colonized adult female *Cx. pipiens* were infected with CxFV by needle inoculation and held for 4, 6, 8 or 12 days. Ovaries were removed and tested by RT-PCR using CxFV-specific primers. CxFV RNA was detected in all samples (Figure 2) demonstrating that CxFV can disseminate to the ovaries within 4 days when administered by the needle route.

Additional experiments were performed using fourth laboratory-generation mosquitoes derived from one of the eight uninfected, field-collected *Cx. pipiens* that oviposited viable egg rafts. Briefly, 100 F_4 adult females were infected with CxFV by needle inoculation. Eleven of these mosquitoes produced viable egg rafts. Subsequent analysis of the parental mosquitoes by RT-PCR using CxFV-specific primers revealed that 9 produced strong positive bands, whereas 2 produced weak positive bands (data not shown). A subset of adult progeny, from the 9 adults that yielded strong positive bands, was assayed in pools of five for CxFV RNA. Two F_4 (denoted as K2 and K9) generated CxFV RNA-positive progeny. Five of the 10 pools derived from K2 were positive as were all 10 pools from K9. All 63 progeny from the remaining 7 F_4 were negative for CxFV RNA. Thus, the extremely high TOT rate observed in the initial studies was not duplicated with the laboratory-colonized needle-inoculated mosquitoes, as the TOT rate for CxFV by this cohort of mosquitoes was 22.2%.

Discussion

The current study provides evidence that efficient transovarial transmission of CxFV by *Cx. pipiens* occurs in the field: the FI and TOT rates for CxFV in naturally-infected adult female mosquitoes were 97.4% and 100%, respectively. These values are considerably greater than the <1% FI and vertical infection rates typically reported in mosquitoes infected with flaviviruses that possess the capacity to replicate in both vertebrates and mosquitoes (Rosen et al. 1978, Aitken et al. 1979, Beaty et al. 1980, Kay and Carley 1980, Tesh 1980, Francy et al. 1981, Hardy et al. 1984). The detection of CxFV RNA in the ovaries of infected *Cx. pipiens* is not surprising in context of the above CxFV TOT data because successful dissemination of the virus to the ovarioles and follicles is required for TOT to occur. Flaviviruses that cycle between vertebrates and mosquitoes, however, rarely disseminate to the ovaries of mosquitoes, consistent with the inefficient rate at which they are vertically transmitted by their arthropod vectors (Turell 1988, Girard et al. 2004, Zhang et al. 2010). Although we have assumed that our findings provide evidence of efficient TOT, it is possible (albeit unlikely) that the high infection rate was instead because of another form of vertical transmission such as transovum transmission. To provide more conclusive evidence that CxFV is maintained in nature by efficient TOT, future experiments should investigate whether CxFV persistently infects the germ line tissues in the ovaries. The efficiency by which *Cx. pipiens* vertically transmits CxFV does not preclude the possibility that the virus is also amplified in the field by other modes of transmission. Indeed, horizontal (i.e. venereal) and/or mechanical (i.e. per os) transmission of CxFV could also occur in nature. In this

regard, larval and adult *Ae. aegypti* (L.) are susceptible to KRV infection *per os* (Lutomiah et al. 2007). Future studies should be performed to identify other routes used by CxFV to infect mosquitoes.

Lutomiah et al. (2007) recently demonstrated vertical transmission of KRV in laboratory-colonized *Ae. aegypti.* In these experiments, female mosquitoes were exposed to KRV by artificial blood meal, subjected to single-pair mating and allowed to oviposit. Thirteen KRV-infected F_0 females were identified. The FI rate in the F_1 produced by these infected mosquitoes after the second and third ovarian cycles was 3.9%. The TOT rate was not reported. One likely explanation for the dramatically lower FI rate in the above study as compared to the FI rate of 97.4% reported in this work is that there is no direct evidence to indicate the *Ae. aegypti* is a natural host of KRV. This virus has only been isolated from *Ae. macintoshi* in the field (Sang et al. 2003) and vertical transmission is presumably more efficient in the natural mosquito host. The lower FI rate could also be attributed to the different method used to assay the F_1 (e.g. virus isolation) or because laboratory-colonized mosquitoes were used. The aforementioned CxFV and KRV studies were performed with *Aedes* spp. mosquitoes from different genera and therefore, the contrasting FI rates could also be due to host differences.

Although our study demonstrated efficient TOT of CxFV by naturally infected *Cx. pipiens*, this virus was not detected in the F_1 of any laboratory-colonized mosquitoes infected by needle inoculation. One explanation for the differential TOT rates between the naturally and experimentally infected mosquitoes could be that the latter mosquitoes are refractory or less susceptible to TOT as a consequence of their long-term

maintenance under laboratory conditions. This could explain the intermediate TOT rate (22.2%) reported in the experiments performed with mosquitoes maintained in the laboratory for only four generations. Alternatively, a subset of mosquitoes, including the majority of the mosquitoes used to establish the short-term (fourth generation) and long-term laboratory colonies, could naturally possess an ovarian escape barrier that renders them refractory to TOT. Another explanation is that mosquitoes with life-long infections (i.e. vertically-infected mosquitoes) may be more susceptible to TOT than mosquitoes infected as adults. For instance, vertical infections could cause long-term pathological manifestations in the ovaries during development that increase susceptibility to efficient vertical passage.

Mosquitoes inoculated with CxFV via the needle route could also be refractory or less susceptible to TOT by virtue of the artificial means by which they were infected. However, this is unlikely given that efficient TOT has been reported in studies performed using mosquitoes infected with bunyaviruses by needle inoculation (Tesh 1980, Turell et al. 1982, Chandler et al. 1990). It is also important to note that administration of CxFV via the needle route does not appear to inhibit viral dissemination to the ovaries as demonstrated by the detection of CxFV RNA in these tissues as early as 4 days post-inoculation. However, it is possible, albeit unlikely, that the RT-PCR results were because of trace amounts of neighboring infected tissue removed with the ovaries rather than successful viral dissemination to the ovaries. Detailed immunohistochemistry studies of needle-inoculated verses F_1 vertically infected mosquitoes using CxFV-specific antibodies will need to be conducted to assess

this theory further. The number of gonotrophic cycles completed by mosquitoes can greatly influence their ability to transovarially transmit virus (Miller et al. 1979, Francy et al. 1981, Anderson et al. 2008). For example, La Crosse virus FI rates of 0, 43 and 58% were reported in *Ae. triseriatus* after the first, second and third ovarian cycles, respectively (Miller et al. 1979). However, this does not explain the differential TOT rates reported in this work because the field and laboratory mosquitoes received equal numbers of blood meals. Nevertheless, it is certainly feasible that TOT of CxFV could have occurred with the laboratory mosquitoes had they been provided with more than one blood meal.

The tissue tropism experiments revealed the presence of CxFV RNA in all of the mosquito organs examined, suggesting that CxFV establishes a systemic infection in the mosquito host. The detection of CxFV RNA in the salivary glands of infected mosquitoes is intriguing because, as a result of the inability of this virus to infect vertebrates, establishment of a salivary gland infection does not appear necessary for the virus to persist in nature. These findings imply that the viral genetic determinants needed for vertebrate-mosquito flaviviruses to disseminate to the salivary glands of their mosquito vectors have been maintained by viruses in the insect-specific lineage. Recently, Kent et al. (2010) demonstrated that CxFV is not secreted into the saliva of infected *Culex quinquefasciatus* Say. These data, together with our findings, could indicate that CxFV replicates poorly in the salivary glands of infected *Culex* spp. mosquitoes, thereby resulting in viral titers that do not support efficient secretion into the saliva. Another explanation is that *Culex* spp. mosquitoes possess a salivary escape

barrier that inhibits the secretion of CxFV into the saliva. Interestingly, however, CxFV was present in the saliva of mosquitoes co-infected with CxFV and WNV (Kent et al. 2010) which implies that, under certain conditions, the potential salivary gland escape barrier can be overcome. Kent et al. (2010) also reported significantly higher WNV transmission rates in mosquitoes infected with both viruses as compared with mosquitoes infected with WNV alone. The potential for exacerbated interactions between WNV and CxFV has been further demonstrated by a study performed in Illinois in which WNV-positive *Cx. pipiens* pools were four times more likely to be infected with CxFV than WNV-negative pools from the same area, and 40% of individual WNV-infected mosquito pools were also CxFV positive (Newman et al. 2011). Thus, despite the apparent inability of CxFV to replicate in vertebrates, this virus could have an indirect negative impact on human and animal health highlighting the important need to further understand the mechanisms by which CxFV is maintained in mosquito populations.

In summary, we provide evidence that efficient TOT of CxFV occurs in naturally infected *Cx. pipiens*. It is likely that other insect-specific flaviviruses use the same strategy to persist in nature but additional research will be required to directly address this issue. Future studies should also investigate whether other forms of transmission are utilized by CxFV to persist in nature.

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References Cited

Aitken, T. H., R. B. Tesh, B. J. Beaty, and L. Rosen. 1979. Transovarial transmission of yellow fever virus by mosquitoes (*Aedes aegypti*). Am J Trop Med Hyg 28: 119-21.

Anderson, J. F., A. J. Main, K. Delroux, and E. Fikrig. 2008. Extrinsic incubation periods for horizontal and vertical transmission of West Nile virus by *Culex pipiens pipiens* (Diptera: Culicidae). J Med Entomol 45: 445-51.

Beaty, B. J., and D. H. Bishop. 1988. Bunyavirus-vector interactions. Virus Res 10: 289-301.

Beaty, B. J., R. B. Tesh, and T. H. Aitken. 1980. Transovarial transmission of yellow fever virus in *Stegomyia* mosquitoes. Am J Trop Med Hyg 29: 125-32.

Blitvich, B. J., M. Lin, K. S. Dorman, V. Soto, E. Hovav, B. J. Tucker, M. Staley, K. B. Platt, and L. C. Bartholomay. 2009. Genomic sequence and phylogenetic analysis of Culex flavivirus, an insect-specific flavivirus, isolated from *Culex pipiens* (Diptera: Culicidae) in Iowa. J Med Entomol 46: 934-41.

Chandler, L. J., B. J. Beaty, G. D. Baldridge, D. H. Bishop, and M. J. Hewlett. 1990. Heterologous reassortment of bunyaviruses in *Aedes triseriatus* mosquitoes and transovarial and oral transmission of newly evolved genotypes. J Gen Virol 71 (Pt 5): 1045-50.

Cook, S., S. N. Bennett, E. C. Holmes, R. De Chesse, G. Moureau, and X. de Lamballerie. 2006. Isolation of a new strain of the flavivirus cell fusing agent virus in a natural mosquito population from Puerto Rico. J Gen Virol 87: 735-48.

Cook, S., G. Moureau, R. Harbach, L. Mukwaya, K. Goodger, F. Ssenfuka, E. Gould, E. C. Holmes, and X. de Lamballerie. 2009. Isolation of a new species of flavivirus and a novel strain of Culex flavivirus (*Flaviviridae*), from a natural mosquito population in Uganda. J Gen Virol.

Crabtree, M. B., P. T. Nga, and B. R. Miller. 2009. Isolation and characterization of a new mosquito flavivirus, Quang Binh virus, from Vietnam. Arch Virol.

Crabtree, M. B., R. C. Sang, V. Stollar, L. M. Dunster, and B. R. Miller. 2003. Genetic and phenotypic characterization of the newly described insect flavivirus, Kamiti River virus. Arch Virol 148: 1095-118.

Farfan-Ale, J. A., M. A. Lorono-Pino, J. E. Garcia-Rejon, V. Soto, M. Lin, M. Staley, K. S. Dorman, L. C. Bartholomay, E. Hovav, and B. J. Blitvich. 2010. Detection of flaviviruses and orthobunyaviruses in mosquitoes in the Yucatan Peninsula of Mexico in 2008. Vector Borne Zoonotic Dis 10: 777-83.

Farfan-Ale, J. A., M. A. Lorono-Pino, J. E. Garcia-Rejon, E. Hovav, A. M. Powers, M. Lin, K. S. Dorman, K. B. Platt, L. C. Bartholomay, V. Soto, B. J. Beaty, R. S. Lanciotti, and B. J. Blitvich. 2009. Detection of RNA from a Novel West Nile-like virus and high prevalence of an insect-specific flavivirus in mosquitoes in the Yucatan Peninsula of Mexico. Am J Trop Med Hyg 80: 85-95.

Francy, D. B., W. A. Rush, M. Montoya, D. S. Inglish, and R. A. Bolin. 1981. Transovarial transmission of St. Louis encephalitis virus by *Culex pipiens* complex mosquitoes. Am J Trop Med Hyg 30: 699-705.

Girard, Y. A., K. A. Klingler, and S. Higgs. 2004. West Nile virus dissemination and tissue tropisms in orally infected *Culex pipiens quinquefasciatus.* Vector Borne Zoonotic Dis 4: 109-22.

Hardy, J. L., L. Rosen, W. C. Reeves, R. P. Scrivani, and S. B. Presser. 1984. Experimental transovarial transmission of St. Louis encephalitis virus by *Culex* and *Aedes* mosquitoes. Am J Trop Med Hyg 33: 166-75.

Hoshino, K., H. Isawa, Y. Tsuda, K. Sawabe, and M. Kobayashi. 2009. Isolation and characterization of a new insect flavivirus from *Aedes albopictus* and *Aedes flavopictus* mosquitoes in Japan. Virology 391: 119-29.

Hoshino, K., H. Isawa, Y. Tsuda, K. Yano, T. Sasaki, M. Yuda, T. Takasaki, M. Kobayashi, and K. Sawabe. 2007. Genetic characterization of a new insect flavivirus isolated from *Culex pipiens* mosquito in Japan. Virology 359: 405-14.

Hughes, M. T., J. A. Gonzalez, K. L. Reagan, C. D. Blair, and B. J. Beaty. 2006. Comparative potential of *Aedes triseriatus*, *Aedes albopictus*, and *Aedes aegypti* (Diptera: Culicidae) to transovarially transmit La Crosse virus. J Med Entomol 43: 757-61.

Huhtamo, E., N. Putkuri, S. Kurkela, T. Manni, A. Vaheri, O. Vapalahti, and N. Y. Uzcategui. 2009. Characterization of a novel flavivirus from mosquitoes in

northern europe that is related to mosquito-borne flaviviruses of the tropics. J Virol 83: 9532-40.

[ICTV] International Committee on Taxonomy of Viruses. 2005.Virus taxonomy: Eighth report of the International Committee on Taxonomy of Viruses Editors: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. Elsevier Academic Press. London.

Junglen, S., A. Kopp, A. Kurth, G. Pauli, H. Ellerbrok, and F. H. Leendertz. 2009. A new flavivirus and a new vector: characterization of a novel flavivirus isolated from *Uranotaenia* mosquitoes from a tropical rain forest. J Virol 83: 4462-8.

Kay, B. H., and J. G. Carley. 1980. Transovarial transmission of Murray Valley encephalitis virus by *Aedes aegypti* (L). Aust J Exp Biol Med Sci 58: 501-4.

Kent, R. J., M. B. Crabtree, and B. R. Miller. 2010. Transmission of West Nile virus by *Culex quinquefasciatus* say infected with Culex Flavivirus Izabal. PLoS Negl Trop Dis 4: e671.

Kihara, Y., T. Satho, Y. Eshita, K. Sakai, A. Kotaki, T. Takasaki, Y. Rongsriyam, N. Komalamisra, R. Srisawat, P. Lapcharoen, S. Sumroiphon, S. Iwanaga, H. Ushijima, D. Endoh, T. Miyata, A. Sakata, N. Kashige, F. Miake, S. Fukushi, M. Saijo, I. Kurane, S. Morikawa, and T. Mizutani. 2007. Rapid determination of viral RNA sequences in mosquitoes collected in the field. J Virol Methods 146: 372-4.

Kim, D. Y., H. Guzman, R. Bueno, Jr., J. A. Dennett, A. J. Auguste, C. V. Carrington, V. L. Popov, S. C. Weaver, D. W. Beasley, and R. B. Tesh. 2009. Characterization of Culex Flavivirus (*Flaviviridae*) strains isolated from mosquitoes in the United States and Trinidad. Virology 386: 154-9.

Lee, J. H., and W. A. Rowley. 2000. The abundance and seasonal distribution of *Culex* mosquitoes in Iowa during 1995-97. J Am Mosq Control Assoc 16: 275-8.

Lutomiah, J. J., C. Mwandawiro, J. Magambo, and R. C. Sang. 2007. Infection and vertical transmission of Kamiti river virus in laboratory bred *Aedes aegypti* mosquitoes. J Insect Sci 7: 1-7.

Miller, B. R., G. R. DeFoliart, and T. M. Yuill. 1979. *Aedes triseriatus* and La Crosse virus: lack of infection in eggs of the first ovarian cycle following oral infection of females. Am J Trop Med Hyg 28: 897-901.

Morales-Betoulle, M. E., M. L. Pineda, S. M. Monzón; Sosa, N. Panella, M. R. LóPEZ B, C. Cordón-Rosales, N. Komar, A. Powers, and B. W. Johnson. 2008. Culex

Flavivirus Isolates from Mosquitoes in Guatemala Journal of Medical Entomology 45: 1187-1190.

Moureau, G., L. Ninove, A. Izri, S. Cook, X. De Lamballerie, and R. N. Charrel. 2009. Flavivirus RNA in Phlebotomine Sandflies. Vector Borne Zoonotic Dis.

Newman, C. M., F. Cerutti, T. K. Anderson, G. L. Hamer, E. D. Walker, U. D. Kitron, M. O. Ruiz, J. D. Brawn, and T. L. Goldberg. 2011. Culex Flavivirus and West Nile Virus Mosquito Coinfection and Positive Ecological Association in Chicago, United States. Vector Borne Zoonotic Dis.

Pabbaraju, K., K. C. Ho, S. Wong, J. D. Fox, B. Kaplen, S. Tyler, M. Drebot, and P. A. Tilley. 2009. Surveillance of mosquito-borne viruses in Alberta using reverse transcription polymerase chain reaction with generic primers. J Med Entomol 46: 640-8.

Rosen, L. 1988. Further observations on the mechanism of vertical transmission of flaviviruses by *Aedes* mosquitoes. Am J Trop Med Hyg 39: 123-6.

Rosen, L., R. B. Tesh, J. C. Lien, and J. H. Cross. 1978. Transovarial transmission of Japanese encephalitis virus by mosquitoes. Science 199: 909-11.

Saiyasombat, R., K. S. Dorman, J. E. Garcia-Rejon, M. A. Lorono-Pino, J. A. Farfan-Ale, and B. J. Blitvich. 2010. Isolation and sequence analysis of Culex flavivirus from *Culex interrogator* and *Culex quinquefasciatus* in the Yucatan Peninsula of Mexico. Arch Virol 155: 983-6.

Sanchez-Seco, M. P., A. Vazquez, X. Collao, L. Hernandez, C. Aranda, S. Ruiz, R. Escosa, E. Marques, M. A. Bustillo, F. Molero, and A. Tenorio. 2009. Surveillance of Arboviruses in Spanish Wetlands: Detection of New Flavi- and Phleboviruses. Vector Borne Zoonotic Dis.

Sang, R. C., A. Gichogo, J. Gachoya, M. D. Dunster, V. Ofula, A. R. Hunt, M. B. Crabtree, B. R. Miller, and L. M. Dunster. 2003. Isolation of a new flavivirus related to cell fusing agent virus (CFAV) from field-collected flood-water *Aedes* mosquitoes sampled from a dambo in central Kenya. Arch Virol 148: 1085-93.

Staley, M., K. S. Dorman, L. C. Bartholomay, I. Fernandez-Salas, J. A. Farfan-Ale, M. A. Lorono-Pino, J. E. Garcia-Rejon, L. Ibarra-Juarez, and B. J. Blitvich. 2010. Universal primers for the amplification and sequence analysis of actin-1 from diverse mosquito species. J Am Mosq Control Assoc 26: 214-8.

Stollar, V., and V. L. Thomas. 1975. An agent in the *Aedes aegypti* cell line (Peleg) which causes fusion of *Aedes albopictus* cells. Virology 64: 367-77.

Tesh, R. B. 1980. Experimental studies on the transovarial transmission of Kunjin and San Angelo viruses in mosquitoes. Am J Trop Med Hyg 29: 657-66.

Turell, M. J. 1988. Horizontal and vertical transmission of viruses by insect and tick vectors, pp. 127-152. *In* T. P. Monath [ed.], The Arboviruses: Epidemiology and Ecology. CRC Press, Boca Raton, FL.

Turell, M. J., W. C. Reeves, and J. L. Hardy. 1982. Evaluation of the efficiency of transovarial transmission of California encephalitis viral strains in *Aedes dorsalis* and *Aedes melanimon.* Am J Trop Med Hyg 31: 382-8.

Tyler, S., B. G. Bolling, C. D. Blair, A. C. Brault, Pabbaraju K., V. M. Armijos, D. C. Clark, and M. A. Drebot. In press. Distribution and phylogenetic comparisons of a novel mosquito flavivirus sequence present in *Culex tarsalis* mosquitoes from western Canada, California and Colorado. The American Journal of Tropical Medicine and Hygiene.

Woodring, J., L. J. Chandler, C. T. Oray, M. M. McGaw, C. D. Blair, and B. J. Beaty. 1998. Short report: Diapause, transovarial transmission, and filial infection rates in geographic strains of La Crosse virus-infected *Aedes triseriatus.* Am J Trop Med Hyg 58: 587-8.

Zhang, M., X. Zheng, Y. Wu, M. Gan, A. He, Z. Li, J. Liu, and X. Zhan. 2010. Quantitative analysis of replication and tropisms of Dengue virus type 2 in *Aedes albopictus.* Am J Trop Med Hyg 83: 700-7.

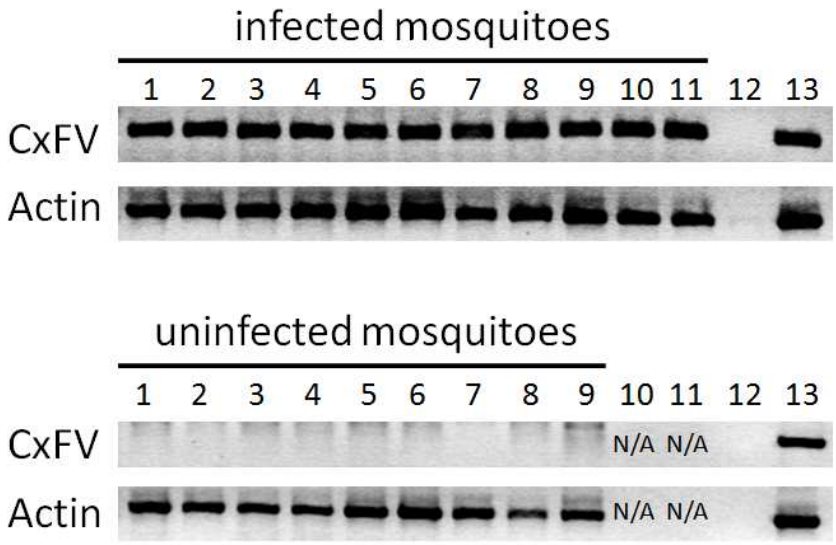

Figure 1. Tissue tropism of CxFV in *Cx. pipiens.* Total RNA was extracted from female and male whole bodies (lanes 1 and 2), female and male midguts (lanes 3 and 4), female and male fat bodies (lanes 5 and 6), female and male heads (lanes 7 and 8), ovaries (lane 9), testes (lane 10) and female salivary glands (lane 11) and assayed by RT-PCR using CxFV and actin-specific primers. Negative and positive control RT-PCRs were included in lanes 12 and 13, respectively. These experiments were performed using F_1 adults from field-collected CxFV RNA-positive *Cx. pipiens* (top panel) and uninfected laboratory-colonized adult mosquitoes (bottom panel), although reproductive organs were not dissected from the latter (denoted as N/A).

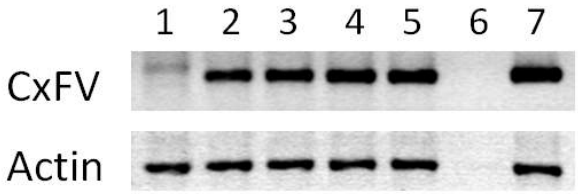

Figure 2. Time-course analysis of CxFV dissemination to the ovaries of infected *Cx. pipiens.* Laboratory-colonized mosquitoes were infected with CxFV by needle inoculation and held for 4, 6, 8 or 12 days (lanes 2 to 5, respectively). Ovaries were dissected and total RNA was extracted and assayed using RT-PCR using CxFV and actin-specific primers. Ovaries from uninfected laboratory-colonized mosquitoes were also tested (lane 1). Negative and positive control RT-PCRs were included (lanes 6 and 7).

Table 1. Culex flavivirus filial infection rates in the F_1 progeny of field-collected CxFV RNA-positive *Cx. pipiens*

Identification number of F_0	Egg raft collection date	Number of CxFV RNA-positive F_1 adults		
		Female	Male	Total
RC9	09/2009	15/15	14/15	29/30
PC31	09/2009	15/15	14/15	29/30
PC39	09/2009	14/15	15/15	29/30
PC96	09/2009	15/15	14/15	29/30
H1	07/2010	15/15	14/15	29/30
H4	07/2010	15/15	15/15	30/30
H6	07/2010	15/15	15/15	30/30
H7	07/2010	14/15	12/15	26/30
H11	07/2010	13/15	14/15	27/30
H18	07/2010	15/15	14/15	29/30
H25	07/2010	15/15	14/15	29/30
H27	07/2010	15/15	15/15	30/30
H32	07/2010	15/15	15/15	30/30
H42	07/2010	15/15	15/15	30/30
H56	07/2010	15/15	15/15	30/30
H61	07/2010	15/15	15/15	30/30
H79	07/2010	15/15	15/15	30/30
I15	08/2010	15/15	15/15	30/30
Total		266/270 (98.5%)	260/270 (96.3%)	526/540 (97.4%)

CHAPTER 3

ISOLATION AND SEQUENCE ANALYSIS OF CULEX FLAVIVIRUS FROM *CULEX INTERROGATOR* AND *CULEX QUINQUEFASCIATUS* IN THE YUCATAN PENINSULA OF MEXICO

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Abstract

Previously, we reported a high prevalence of Culex flavivirus (CxFV) in *Culex quinquefasciatus* (Say) in the Yucatan Peninsula of Mexico. To determine whether other *Culex* spp. mosquitoes in this region are susceptible to natural CxFV infection, *Cx. bahamensis* (Dyar and Knab), *Cx. coronator* (Dyar and Knab), *Cx. interrogator* (Dyar and Knab), *Cx. nigripalpus* (Theobald) and *Cx. opisthopus* (Komp) in the Yucatan Peninsula of Mexico were tested for CxFV. Two pools of *Cx. interrogator* were positive. The envelope protein genes of these isolates and 16 isolates from *Cx. quinquefasciatus*

were sequenced and shown to have ≥99.2% nucleotide identity. These data suggest that there is limited genetic diversity among CxFV isolates in Yucatan Peninsula of Mexico.

Keywords: Flavivirus, Culex flavivirus, Mosquito, Phylogeny, Mexico

Brief Report

Culex flavivirus (family *Flaviviridae*, genus *Flavivirus*) is an insect-specific virus that was first isolated from *Cx. pipiens* (Linnaeus), *Cx. quinquefasciatus* (Say) and *Cx. tritaeniorhynchus* (Giles) collected in Japan and Indonesia in 2003 and 2004 (Hoshino et al. 2007). More recently, CxFV was detected in *Cx. quinquefasciatus* in Guatemala (Morales-Betoulle et al. 2008), the Yucatan Peninsula of Mexico (Farfan-Ale et al. 2009), Trinidad (Kim et al. 2009) and Uganda (Cook et al. 2009), as well as *Cx. quinquefasciatus* and *Cx. restuans* (Theobald) in Texas (Kim et al. 2009), and *Cx. pipiens* and *Cx. tarsalis* (Coquillet) in Iowa (Blitvich et al. 2009). CxFV replicates in *Aedes albopictus* (C6/36) cells but not in African Green Monkey Kidney (Vero) cells, Baby Hamster Kidney cells or intracerebrally inoculated newborn mice which suggests that this virus is insect-specific.

Eight other insect-specific flaviviruses have been isolated from mosquitoes: cell fusing agent virus (Stollar and Thomas 1975, Cook et al. 2006, Kihara et al. 2007), Kamiti River virus (Crabtree et al. 2003, Sang et al. 2003), Quang Binh virus (Crabtree et al. 2009), Aedes flavivirus (Hoshino et al. 2009), Nakiwogo virus (Cook et al. 2009) Lammi virus (Huhtamo et al. 2009), Nouname virus (Junglen et al. 2009) and Calbertado virus (M. A. Drebot, personal communication). A potentially novel insect-specific

flavivirus (es) has also been identified in *Phlebotomine* sandflies (Moureau et al. 2009, Sanchez-Seco et al. 2009). In addition, flavivirus-related DNA known as cell silent agent is integrated into the genomes of some *Aedes* spp. mosquitoes (Crochu et al. 2004, Roiz et al. 2009).

There is a current lack of information of the host range and genetic diversity of CxFV in Mexico. The prototype Mexican strain of CxFV (designated CxFV-Mex07), which was isolated from *Cx. quinquefasciatus* in the Yucatan Peninsula of Mexico in 2007, represents the only CxFV isolate from Mexico for which sequence data are available (Farfan-Ale et al. 2009). Furthermore, *Cx. quinquefasciatus* is the only mosquito species from which the Mexican strain of CxFV has been isolated (Farfan-Ale et. al., 2009, Farfan-Ale et. al., 2010). In this report, all *Culex* spp. mosquitoes (with the exception of *Cx. quinquefasciatus*) that had been collected in our mosquito-based virus surveillance in the Yucatan Peninsula of Mexico in 2008 (Farfan-Ale et al. 2010) were assayed by reverse transcription-polymerase chain reaction (RT-PCR) using flavivirus- and CxFV-specific primers. All of these mosquitoes had previously been tested for cytopathic virus by virus isolation in Vero cells. The purpose of our present study was to identify other insect-specific flaviviruses that may be present in the Yucatan Peninsula of Mexico and to increase our knowledge on the host range and genetic diversity of CxFV in this region.

Culex spp. mosquitoes tested in this study were collected at study sites in Isla Mujeres, Merida, Sian Ka'an and Tixkokob in the Yucatan Peninsula of Mexico. Detailed descriptions of these study sites and the protocols used for the collection,

identification and homogenization of mosquitoes have been provided elsewhere (Darsie 1996, Farfan-Ale et al. 2009, Farfan-Ale et al. 2010). Total RNA was extracted from mosquito homogenates using the QIAamp viral RNA extraction kit (QIAGEN, Valencia, CA). Complementary DNAs were generated using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) and PCRs were performed using *Taq* polymerase (Invitrogen, Carlsbad, CA) and flavivirus- and CxFV-specific primers. The flavivirus-specific primers, FU2 and cFD3, target a 845-nt region of the NS5 gene (Kuno et al. 1998). The CxFV-specific primers, CxFV(E)-PCR-F (5'-ACTGGTGACGTTCAAGGCCATAAG-3') and CxFV(E)-PCR-R (5'-GCCGTGATCAGGTGCTGGTCATCG-3') target a 1,551-nt region of the CxFV genome that includes the entire (1,281-nt) envelope (E) protein gene. RT-PCR products were purified using a Purelink Gel Extraction Kit (Invitrogen, Carlsbad, CA) and sequenced using a 3730x1 DNA sequencer (Applied Biosystems, Foster City, CA). Sequencing reactions were performed using 12 CxFV-specific primers, and primer sequences are available upon request.

A total of 1,856 *Culex* spp. mosquitoes in 121 pools were tested by RT-PCR. The mosquitoes belong to 5 species: *Cx. bahamensis* (Dyar and Knab) (n = 3), *Cx. coronator* (Dyar and Knab) (n = 154), *Cx. interrogator* (Dyar and Knab) (n = 766), *Cx. nigripalpus* (Theobald) (n = 235) and *Cx. opisthopus* (Komp) (n = 698). Of these, 1,805 (97.3%) were identified as female and 51 (2.7%) as male. Two pools of female *Cx. interrogator* were positive by RT-PCR using flavivirus- and CxFV-specific primers. All other mosquitoes were negative with both primer pairs. However, due to the small numbers of *Cx. bahamensis*, *Cx. coronator*, *Cx. nigripalpus* and *Cx. opisthopus* available for testing,

it would be premature to conclude that these species are not natural hosts of CxFV until more research is done to address this issue. The CxFV minimal infection rate (MIR), expressed as the number of positive mosquito pools per 1,000 mosquitoes tested, in *Cx. interrogator* was 2.6. Because of the considerable variation in pool sizes, bias corrected maximum likelihood estimation (MLE) values were also calculated using the PooledInfRate statistical software package (Biggerstaff 2006). The MLE value (and 95% confidence interval) for CxFV in *Cx. interrogator* was 2.6 (0.48 – 8.42). One pool positive for CxFV RNA was collected in Tixkokob in January 2008; the other pool was collected in Merida in June 2008. Previously, we reported a much higher CxFV MIR in *Cx. quinquefasciatus* in the Yucatan Peninsula of Mexico (Farfan-Ale et al. 2009, Farfan-Ale et al. 2010). The CxFV MIRs in *Cx. quinquefasciatus* collected in Merida and Tixkokob from June through August 2007 were 10.9 and 26.0, respectively. The overall CxFV MIR in *Cx. quinquefasciatus* collected in Merida from January to December 2008 was 7.7, and the monthly MIRs ranged from 4.3 to 16.6. Previously, we also reported that CxFV RNA was not detected by RT-PCR in any *Aedes*, *Anopheles*, *Ochlerotatus*, *Mansonia* and *Psorophora* spp. mosquitoes tested (Farfan-Ale et al. 2010).

The two mosquito homogenates that were positive for CxFV RNA were tested for virus by virus isolation in C6/36 cells as described previously (Farfan-Ale et al. 2009). CxFV was isolated from both homogenates. The isolate from Tixkokob has been designated T-955, and the isolate from Merida have been designated M-2168. Twenty homogenates that had previously tested positive for CxFV RNA by RT-PCR (Farfan-Ale

et al. 2010) were also processed by virus isolation in C6/36 cells. Sixteen isolates were obtained, and all are from *Cx. quinquefasciatus* collected in Merida in 2008.

The E protein genes of the 2 CxFV isolates from *Cx. interrogator* as well as the 16 CxFV isolates from *Cx. quinquefasciatus* were sequenced (Genbank Accession nos. GU289683 to GU289700). Pairwise alignments of the nucleotide sequences using the CLUSTAL W algorithm (version 2) (Higgins and Sharp 1988, Larkin et al. 2007) revealed that they have 99.2 to 100% identity. Of the 1,280 nucleotides that comprise the E protein gene, 1,245 (97.3%) are strictly conserved between all isolates. The deduced amino acid sequences have 99.5 to 100% identity, and 99.5 to 100% similarity. In total, 419 of the 426 (98.4%) amino acid positions are strictly conserved between these isolates. The nucleotide and deduced amino acid sequences of these isolates were also aligned to the homologous region of the prototype Mexican strain of this virus, CxFV-Mex07, which was isolated from *Cx. quinquefasciatus* in Tixkokob in 2007 (Farfan-Ale et al. 2009) (Genbank Accession number EU879060). The E protein gene of CxFV-Mex07 has at least 99.5% nucleotide identity, 99.5% amino acid identity and 99.8% amino acid similarity to the 18 E protein gene sequences from in this study. These findings suggest that there is minimal genetic diversity between CxFV isolates in the Yucatan Peninsula of Mexico.

A phylogenetic tree was constructed with Bayesian methods using the E protein gene sequences of 34 CxFV isolates, including the 18 isolates obtained in this study (Figure 1). The analysis revealed that all isolates from this study have a close phylogenetic relationship with CxFV-Mex07, in addition to CxFV isolates from

Guatemala, Trinidad and Uganda. These isolates comprise a distinct clade (denoted as clade 1). CxFV isolates from the United States and Asia comprise a second clade (denoted as clade 2). The posterior support for the branch separating these two clades is 100%. The Mexican isolates form a monophyletic group within clade 1, and the posterior support for this topological arrangement is 84%. Phylogenetic trees were also generated using neighbor-joining, maximum parsimony and maximum likelihood methods, and all of these trees showed the same topological features as the Bayesian tree (data not shown).

In summary, we demonstrate that the host-range of CxFV in the Yucatan Peninsula of Mexico is not restricted to *Cx. quinquefasciatus* and provide evidence of limited genetic and phylogenetic diversity between CxFV isolates in this region. Comparative studies between insect-specific viruses and mosquito-vertebrate flaviviruses will provide important insight into flavivirus evolution and will help us understand why some flaviviruses possess the capacity to replicate and cause disease in humans and vertebrate animals while others do not.

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References

Biggerstaff, B. 2006. Software for mosquito surveillance (cited February 15, 2010). Centers for Disease Control and Prevention, Fort Collins, Colorado. Available from http://www.cdc.gov/ncidod/dvbid/westnile/software.htm

Blitvich, B. J., M. Lin, K. S. Dorman, V. Soto, E. Hovav, B. J. Tucker, M. Staley, K. B. Platt, and L. C. Bartholomay. 2009. Genomic sequence and phylogenetic analysis of Culex flavivirus, an insect-specific flavivirus, isolated from *Culex pipiens* (Diptera: Culicidae) in Iowa. J Med Entomol 46: 934-41.

Cook, S., S. N. Bennett, E. C. Holmes, R. De Chesse, G. Moureau, and X. de Lamballerie. 2006. Isolation of a new strain of the flavivirus cell fusing agent virus in a natural mosquito population from Puerto Rico. J Gen Virol 87: 735-48.

Cook, S., G. Moureau, R. Harbach, L. Mukwaya, K. Goodger, F. Ssenfuka, E. Gould, E. C. Holmes, and X. de Lamballerie. 2009. Isolation of a new species of flavivirus and a novel strain of Culex flavivirus (*Flaviviridae*), from a natural mosquito population in Uganda. J Gen Virol. 90: 2669-78.

Crabtree, M. B., P. T. Nga, and B. R. Miller. 2009. Isolation and characterization of a new mosquito flavivirus, Quang Binh virus, from Vietnam. Arch Virol. 154: 857-60.

Crabtree, M. B., R. C. Sang, V. Stollar, L. M. Dunster, and B. R. Miller. 2003. Genetic and phenotypic characterization of the newly described insect flavivirus, Kamiti River virus. Arch Virol 148: 1095-118.

Crochu, S., S. Cook, H. Attoui, R. N. Charrel, R. De Chesse, M. Belhouchet, J. J. Lemasson, P. de Micco, and X. de Lamballerie. 2004. Sequences of flavivirus-related RNA viruses persist in DNA form integrated in the genome of *Aedes* spp. mosquitoes. J Gen Virol 85: 1971-80.

Darsie, R. F., Jr. 1996. A survey and bibliography of the mosquito fauna of Mexico (Diptera: Culicidae). J Am Mosq Control Assoc 12: 298-306.

Farfan-Ale, J. A., M. A. Lorono-Pino, J. E. Garcia-Rejon, V. Soto, M. Lin, M. Staley, K. S. Dorman, L. C. Bartholomay, E. Hovav, and B. J. Blitvich. 2010. Detection of flaviviruses and orthobunyaviruses in mosquitoes in the Yucatan Peninsula of Mexico in 2008. Vector Borne Zoonotic Dis. (In press).

Farfan-Ale, J. A., M. A. Lorono-Pino, J. E. Garcia-Rejon, E. Hovav, A. M. Powers, M. Lin, K. S. Dorman, K. B. Platt, L. C. Bartholomay, V. Soto, B. J. Beaty, R. S. Lanciotti, and B. J. Blitvich. 2009. Detection of RNA from a Novel West Nile-

like virus and high prevalence of an insect-specific flavivirus in mosquitoes in the Yucatan Peninsula of Mexico. Am J Trop Med Hyg 80: 85-95.

Higgins, D. G., and P. M. Sharp. 1988. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene 73: 237-44.

Hoshino, K., H. Isawa, Y. Tsuda, K. Sawabe, and M. Kobayashi. 2009. Isolation and characterization of a new insect flavivirus from *Aedes albopictus* and *Aedes flavopictus* mosquitoes in Japan. Virology 391: 119-29.

Hoshino, K., H. Isawa, Y. Tsuda, K. Yano, T. Sasaki, M. Yuda, T. Takasaki, M. Kobayashi, and K. Sawabe. 2007. Genetic characterization of a new insect flavivirus isolated from *Culex pipiens* mosquito in Japan. Virology 359: 405-14.

Huhtamo, E., N. Putkuri, S. Kurkela, T. Manni, A. Vaheri, O. Vapalahti, and N. Y. Uzcategui. 2009. Characterization of a novel flavivirus from mosquitoes in northern europe that is related to mosquito-borne flaviviruses of the tropics. J Virol 83: 9532-40.

Junglen, S., A. Kopp, A. Kurth, G. Pauli, H. Ellerbrok, and F. H. Leendertz. 2009. A new flavivirus and a new vector: characterization of a novel flavivirus isolated from *Uranotaenia* mosquitoes from a tropical rain forest. J Virol 83: 4462-8.

Kihara, Y., T. Satho, Y. Eshita, K. Sakai, A. Kotaki, T. Takasaki, Y. Rongsriyam, N. Komalamisra, R. Srisawat, P. Lapcharoen, S. Sumroiphon, S. Iwanaga, H. Ushijima, D. Endoh, T. Miyata, A. Sakata, N. Kashige, F. Miake, S. Fukushi, M. Saijo, I. Kurane, S. Morikawa, and T. Mizutani. 2007. Rapid determination of viral RNA sequences in mosquitoes collected in the field. J Virol Methods 146: 372-4.

Kim, D. Y., H. Guzman, R. Bueno, Jr., J. A. Dennett, A. J. Auguste, C. V. Carrington, V. L. Popov, S. C. Weaver, D. W. Beasley, and R. B. Tesh. 2009. Characterization of Culex Flavivirus (*Flaviviridae*) strains isolated from mosquitoes in the United States and Trinidad. Virology 386: 154-9.

Kuno, G., G. J. Chang, K. R. Tsuchiya, N. Karabatsos, and C. B. Cropp. 1998. Phylogeny of the genus *Flavivirus*. J Virol 72: 73-83.

Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson, and D. G. Higgins. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947-8.

Morales-Betoulle, M. E., M. L. Monzon Pineda, S. M. Sosa, N. Panella, M. R. Lopez, C. Cordon-Rosales, N. Komar, A. Powers, and B. W. Johnson. 2008. Culex flavivirus isolates from mosquitoes in Guatemala. J Med Entomol 45: 1187-90.

Moureau, G., L. Ninove, A. Izri, S. Cook, X. De Lamballerie, and R. N. Charrel. 2009. Flavivirus RNA in *Phlebotomine* Sandflies. Vector Borne Zoonotic Dis.

Roiz, D., A. Vazquez, M. P. Seco, A. Tenorio, and A. Rizzoli. 2009. Detection of novel insect flavivirus sequences integrated in *Aedes albopictus* (Diptera: Culicidae) in Northern Italy. Virol J 6: 93.

Ronquist, F., and J. P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572-4.

Sanchez-Seco, M. P., A. Vazquez, X. Collao, L. Hernandez, C. Aranda, S. Ruiz, R. Escosa, E. Marques, M. A. Bustillo, F. Molero, and A. Tenorio. 2009. Surveillance of Arboviruses in Spanish Wetlands: Detection of New Flavi- and Phleboviruses. Vector Borne Zoonotic Dis.

Sang, R. C., A. Gichogo, J. Gachoya, M. D. Dunster, V. Ofula, A. R. Hunt, M. B. Crabtree, B. R. Miller, and L. M. Dunster. 2003. Isolation of a new flavivirus related to cell fusing agent virus (CFAV) from field-collected flood-water *Aedes* mosquitoes sampled from a dambo in central Kenya. Arch Virol 148: 1085-93.

Stollar, V., and V. L. Thomas. 1975. An agent in the *Aedes aegypti* cell line (Peleg) which causes fusion of *Aedes albopictus* cells. Virology 64: 367-77.

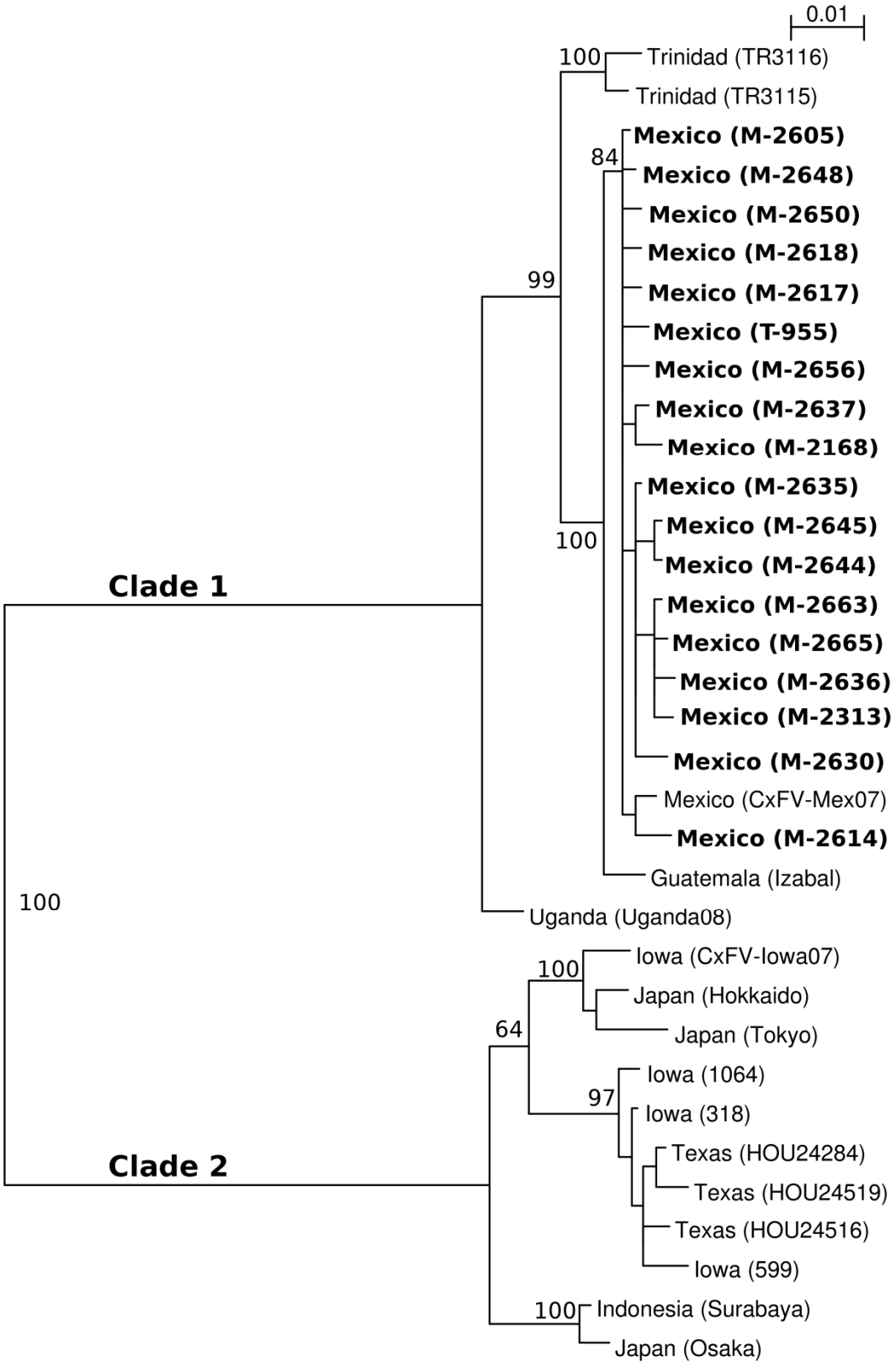
0.01
100
Trinidad (TR3116)
Trinidad (TR3115)
Mexico (M-2605)
84
Mexico (M-2648)
Mexico (M-2650)
Mexico (M-2618)
99
Mexico (M-2617)
Mexico (T-955)
Mexico (M-2656)
Mexico (M-2637)
Mexico (M-2168)
Mexico (M-2635)
Mexico (M-2645)
100
Mexico (M-2644)
Clade 1
Mexico (M-2663)
Mexico (M-2665)
Mexico (M-2636)
Mexico (M-2313)
Mexico (M-2630)
Mexico (CxFV-Mex07)
Mexico (M-2614)
Guatemala (Izabal)
100
Uganda (Uganda08)
Iowa (CxFV-Iowa07)
100
Japan (Hokkaido)
64
Japan (Tokyo)
Iowa (1064)
97
Iowa (318)
Texas (HOU24284)
Clade 2
Texas (HOU24519)
Texas (HOU24516)
Iowa (599)
100
Indonesia (Surabaya)
Japan (Osaka)

Figure 1. Phylogenetic analysis of the envelope protein gene of 18 CxFV isolates obtained in this study and 16 other CxFV isolates. The envelope protein gene encompasses nucleotide positions 938 to 2,217 relative to the genomic sequence of the prototype CxFV strain (Tokyo). The displayed phylogeny was estimated by using the program MRBAYES, version 3.1 (Ronquist and Huelsenbeck 2003). Posterior support (out of 100) for selected branches is indicated. An unrooted tree was inferred but is shown rooted using the midpoint method. CxFV isolates obtained in the present study are denoted in *bold*. GenBank accession numbers for sequences used in the phylogenetic analysis are: M-2168 (GU289684), M-2313 (GU289685), M-2605 (GU289686), M-2614 (GU289687), M-2617 (GU289688), M-2618 (GU289689), M-2630 (GU289690), M-2635 (GU289691), M-2636 (GU289692), M-2637 (GU289693), M-2644 (GU289694), M-2645 (GU289695), M-2648 (GU289696), M-2650 (GU289697), M-2656 (GU289698), M-2663 (GU289699), M-2665 (GU289700), T-955 (GU289683), Mex07 (EU879060.1), Guatemala (EU805805.1), Iowa07 (FJ663034.1), Iowa 1064 (FJ663026.1), Iowa 318 (FJ663030.1), Iowa 599 (FJ663032.1), HOU24284 (FJ502997.1), HOU24519 (FJ502996.1), HOU24516 (FJ502999.1), Hokkaido (AB262762.1), Osaka (AB262763.1), Surabaya (AB262766.1), Tokyo (AB262759.2), TR3115 (FJ503002.1), TR3116 (FJ503003.1) and Uganda (GQ165808.1).

CHAPTER 4

SUBSTITUTION OF THE PREMEMBRANE AND ENVELOPE PROTEIN GENES OF MODOC VIRUS WITH THE HOMOLOGOUS SEQUENCES OF WEST NILE VIRUS GENERATES A CHIMERIC VIRUS THAT REPLICATES IN VERTEBRATE BUT NOT MOSQUITO CELLS

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Abstract

Background: Most known flaviviruses, including West Nile virus (WNV), are maintained in natural transmission cycles between hematophagous arthropods and vertebrate hosts. Other flaviviruses such as Modoc virus (MODV) and Culex flavivirus (CxFV) have host ranges restricted to vertebrates and insects, respectively. The genetic elements that modulate the differential host ranges and transmission cycles of these viruses have not been identified.

Methods: Fusion polymerase chain reaction (PCR) was used to replace the capsid (C), premembrane (prM) and envelope (E) genes and the prM-E genes of a full-length MODV infectious cDNA clone with the corresponding regions of WNV and CxFV. Fusion products were directly transfected into baby hamster kidney-derived cells that stably express T7 RNA polymerase. At 4 days post-transfection, aliquots of each supernatant were inoculated onto vertebrate (BHK-21 and Vero) and mosquito (C6/36) cells which were then assayed for evidence of viral infection by reverse transcription-PCR, Western blot and plaque assay.

Results: Chimeric virus was recovered in cells transfected with the fusion product containing the prM-E genes of WNV. The virus could infect vertebrate but not mosquito cells. The *in vitro* replication kinetics and yields of the chimeric virus were similar to MODV but the chimeric virus produced larger plaques. Chimeric virus was not recovered in cells transfected with any of the other fusion products.

Conclusions: Our data indicate that genetic elements outside of the prM-E gene region of MODV condition its vertebrate-specific phenotype.

Keywords: Modoc virus, West Nile virus, Culex flavivirus, Chimeric flavivirus, Fusion PCR

Introduction

All viruses in the genus *Flavivirus* (family *Flaviviridae*) possess a single-stranded, positive-sense RNA genome of approximately 11 kb (Lindenbach et al., 2007). The genome contains a single open reading frame (ORF) flanked by 5’ and 3’

untranslated regions (UTRs) of ~100 and 400-700 nt, respectively (Markoff, 2003). The 5’ end of the genome contains a type I cap structure and the 3’ end is non-polyadenylated. The ORF encodes a single polyprotein that is co- and post-translationally cleaved to generate three structural proteins, designated the capsid (C), premembrane/membrane (prM/M) and envelope (E) proteins, and at least seven non-structural (NS) proteins in the gene order: 5′–C–prM(M)–E–NS1–NS2A–NS2B–NS3–NS4A–NS4B–NS5-3′ (Lindenbach et al., 2007; Rice et al., 1985). Cleavage events are mediated by a combination of endoplasmic reticulum signalases, furin and the viral trypsin-like serine protease (Falgout et al., 1991; Lindenbach et al., 2007; Stadler et al., 1997).

The flavivirus genome is packaged in an icosahedral nucleocapsid with multiple copies of the C protein (Lindenbach et al., 2007). The nucleocapsid is surrounded by a lipid envelope, acquired from the host cell, in which the prM(M) and E proteins are embedded. The E protein is required for receptor binding, host membrane fusion and viral assembly, while the prM protein protects the E protein from undergoing an irreversible conformational change as the virion is secreted through acidified sorting compartments (Chen et al., 1996; Guirakhoo et al., 1992; Heinz et al., 1994; Rey et al., 1995). RNA replication occurs in the cytoplasm in close association with the rough endoplasmic reticulum and requires the participation of several NS proteins including the viral helicase and protease (NS3), viral protease cofactor (NS2B) and RNA-dependent RNA polymerase and methyltransferase (NS5) (Egloff et al., 2002; Falgout et al., 1991; Tan et al., 1996).

Flaviviruses can be divided into three distinct groups based upon their mode of transmission (Cook et al., 2012; Kuno, 2007). The first group is comprised of viruses that are transmitted horizontally between hematophagous arthropods and vertebrate hosts. This group can be further divided into mosquito-borne and tick-borne viruses. Examples of mosquito-borne flaviviruses include West Nile virus (WNV), dengue virus (DENV), yellow fever virus (YFV) and Japanese encephalitis virus (JEV), all of which are human pathogens of global concern (Gubler et al., 2007). Tick-borne flaviviruses associated with serious human disease include tick-borne encephalitis virus (TBEV), Langat virus (LGTV) and Powassan virus. Flaviviruses in the second group have no known arthropod vector (NKV) and are considered to be vertebrate-specific. NKV flaviviruses have been isolated exclusively from bats and rodents, and examples include Modoc virus (MODV) and Rio Bravo virus (Burns and Farinacci, 1956; Johnson, 1967). The mechanism(s) by which NKV flaviviruses are maintained in nature is poorly defined but it has been suggested that they are transmitted between hosts by nasal and/or oral contact (Bell and Thomas, 1964; Constantine and Woodall, 1964; Zarnke and Yuill, 1985). The final group is comprised of insect-specific flaviviruses (ISFs). These viruses are assumed to be insect-specific because they have been isolated from mosquitoes but do not replicate in mice or any vertebrate cell lines that have been tested. More than 20 ISFs have been discovered including Culex flavivirus (CxFV), cell fusing agent virus and Kamiti River virus (Crabtree et al., 2003; Haddow et al., 2013; Hoshino et al., 2007; Stollar and Thomas, 1975). Recent data indicate that ISFs are maintained in nature by transovarial transmission (Saiyasombat et al., 2011). It is not known whether ISFs and

NKV flaviviruses were originally arthropod-vertebrate flaviviruses that lost the ability to replicate in one host or if they are progenitor viruses from which the arthropod/vertebrate flaviviruses evolved, although the latter theory is favored (Gould et al., 2003; Kuno et al., 1998).

The evolutionary processes and underlying genetic basis for the differential host ranges and transmission cycles of flaviviruses have not been identified. Thus, the overall goal of this study is to characterize the *in vitro* host ranges of chimeric viruses constructed using representative viruses from the vertebrate-specific, insect-specific and arthropod/vertebrate flavivirus groups (MODV, CxFV and WNV, respectively) in order to increase our knowledge of the genetic elements that condition the vastly different host ranges and transmissibilities of these viruses.

Materials and methods

Cell lines

BSR-T7/5 cells, which are baby hamster kidney-derived cells that constitutively express T7 RNA polymerase (Buchholz et al., 1999), were kindly provided by Cathy Miller (Iowa State University). Baby hamster kidney (BHK-21), African Green Monkey kidney (Vero) and *Aedes albopictus* (C6/36) cells were obtained from the American Type Culture Collection (Manassas, VA). BSR-T7/5 and BHK-21 cells were cultured in minimum essential medium (Invitrogen, Carlsbad, CA), Vero cells were cultured in Dulbecco's modified Eagle medium (Invitrogen) and C6/36 cells were cultured in Liebovitz L15 medium (Invitrogen). All media was supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin.

Mammalian cells were cultured at 37°C with 5% CO_2 whereas C6/36 cells were cultured at 28°C.

Viruses

pACNR-FLMODV, which contains full-length cDNA of MODV (strain M544) downstream of a T7 Φ2.5 promoter (Peter J. Bredenbeek, unpublished data), was used as template for fusion PCRs reactions. The plasmid was also used to amplify the full-length product needed to generate MODV. WNV (strain NY99-flamingo382-99) was kindly provided by Aaron Brault (Centers for Disease Control and Prevention). CxFV (strain Iowa07) was originally isolated from *Culex pipiens* in Iowa in 2007 (Blitvich, 2009). cDNAs were generated from WNV and CxFV RNA and used as template for fusion PCR reactions as described below.

Construction of chimeric cDNAs

Four full-length chimeric flavivirus fusion products, designated fpMODV-WNV(C-prM-E), fpMODV-WNV(prM-E), fpMODV-CxFV(C-prM-E) and fpMODV-CxFV(prM-E), were generated by replacing the C-prM-E and prM-E genes of MODV with the homologous genes of WNV and CxFV. Four conventional PCRs and three fusion-PCRs were required to generate each full-length fusion product (Table 1). The process was facilitated by chimeric primers (half MODV sequence and half heterologous virus sequence) that worked as linkers to fuse the intermediate reaction products and subsequently assemble the final chimeras. The strategy used to construct fpMODV-WNV(prM-E) is depicted in Figure 1 and described below as an example of the chimeric viral cDNA construction process. In the first reaction, a 523 bp product (designated

MW1) was amplified by PCR using pACNR-FLMODV as template, a forward primer (M-F1; see Tables 1 and 2) specific to the vector sequence upstream of the MODV 5'UTR and a chimeric reverse primer (MWi-R1) specific to the distal 3' and 5' ends of the MODV C and WNV prM genes, respectively. In the second reaction, a 2066 bp product (MW2) that contains the entire prM-E genes of WNV was amplified by RT-PCR using total RNA extracted from WNV-infected C6/36 cells as template, a forward chimeric primer (MWi-F2) specific to the sequences at the distal 3' and 5' ends of the MODV C and WNV prM genes, respectively and a reverse chimeric primer (MW-R2) specific to the sequences at the distal 3' and 5' ends of the WNV E and MODV NS1 genes, respectively. In the third reaction, a 2575 bp product (MW3) that contains the entire NS1-NS2A-NS2B genes and part of the NS3 gene of MODV was amplified by PCR using pACNR-FLMODV as template, a forward chimeric primer (MW-F3) specific to the WNV E and MODV NS1 genes and a reverse primer (M-R3) specific to an internal region of the MODV NS3 gene. In reaction four, the remainder of the NS3 gene and the entire NS4A-NS4B-NS5-3'UTR region of MODV was amplified by PCR using pACNR-FLMODV as template and MODV-specific forward and reverse primers (M-F4 and M-R10600, respectively) to give a 6227 bp product (M4). Reaction 5 was a fusion-PCR in which MW1 and MW2 were used as templates and M-F1 and MW-R2 as primers for the generation of a 2542 bp product designated MW5. Reaction 6 was another fusion-PCR in which MW3 and MW5 were used as templates and M-F1 and M-R3 as primers for the generation of a 5079 bp product designated MW6. In the final reaction, a full-length 10,708 bp chimeric fusion product designated fpMODV-

WNV(prM-E) was generated by fusion-PCR using M4 and MW6 as templates and T7-MOD-F and M-R10600 as forward and reverse primers, respectively. The 5' end of T7-MOD-F contains the T7 promoter sequence. A similar strategy was adopted for the construction of fpMODV-WNV(C-prM-E), fpMODV-CxFV(C-prM-E) and fpMODV-CxFV(prM-E) with the primers used in these experiments and the sizes of the resulting amplification products denoted in Tables 1 and 2. Full-length MODV was also amplified in a single PCR using pACNR-FLMODV as template, T7-MOD-F as the forward primer and M-R10600 as the reverse primer (Table 2). All full-length products were purified by phenol/chloroform extraction and ethanol precipitation, and sequenced using overlapping primers for junction verification.

Transfections and virus recovery

Full-length PCR products (chimeras and full-length MODV) were transfected directly into BSR-T7/5 cells (which stably express T7 RNA polymerase) in order to avoid the *in vitro* transcription step. BSR-T7/5 cells were seeded into 60 mm^2 sterile plates and incubated until there were approximately $9.5x10^5$ cells per plate. Cells were transfected with 5 µg of purified full-length flavivirus cDNA mixed with 500 µl of serum-free Opti-MEM (Invitrogen) and 15 µl of TransIT-LT1 transfection reagent (Mirus Bio, Wisconsin) according to the manufacturer's instructions. Transfected BSR-T7/5 cells were incubated for 4 days then aliquots of each supernatant were collected and inoculated onto subconfluent monolayers of Vero, BHK-21 and C6/36 cells. Several more passages were performed in the same cell type or, where specified, an alternate cell type. Cells were monitored daily for cytopathic effect (CPE). Cell monolayers and

supernatants were harvested when 50-70% of the cells exhibited CPE. If CPE was not observed, cells were harvested at 7 to 9 days post-inoculation (p.i.), with the exception of BHK-21 cells which were harvested at 4 days p.i. since all BHK-21 cell cultures (including the negative control cultures) displayed considerable cell death at this time.

Reverse transcription-polymerase chain reaction

Total RNA was extracted from cell monolayers and supernatants using Trizol Reagent (Invitrogen) and the QIAamp viral RNA mini kit (Qiagen, Valencia, CA), respectively. Complementary DNAs were generated using Superscript III reverse transcriptase (Invitrogen). Where specified, RNA templates were treated with deoxyribonuclease I (DNase I; Invitrogen) prior to reverse transcriptions. PCRs were performed using high fidelity *Taq* polymerase (Invitrogen). MODV, WNV and CxFV-specific primers were designed using published sequences (Genbank Accession No. AJ242984, AF196835 and FJ663034, respectively). PCR products were examined by 0.8-1% agarose gel electrophoresis, purified using QIAquick spin columns (Qiagen) and sequenced using a 3730x1 DNA sequencer (Applied Biosystems, Foster City, CA).

Preparation of protein lysates

BHK-21, Vero and C6/36 cell monolayers, approaching confluency in 75 cm^2 flasks, were inoculated with parental or chimeric virus at a multiplicity of infection (m.o.i) of 0.1 and incubated for 4 days (BHK-21 cells) or 7 days (Vero and C6/36 cells). Cells were scraped from the surface of the flask, clarified by centrifugation (10□000g, 10 min, 4°C), washed twice with cold phosphate-buffered saline (PBS), resuspended in lysing buffer [10 mM Tris–HCl pH 7.5, 150 mM NaC1, 5 mM EDTA, 1% sodium

deoxycholate, 1% Triton X-100, 0.1% SDS and a cocktail of protease inhibitors (Sigma, St. Louis, MO)] and placed on ice for 15 min. Samples were microfuged at 4 °C for 15 min and supernatants collected and stored at -70°C.

Western blots

Protein samples were mixed with an equal volume of reducing sample buffer, heated (95^{o}C for 5 min) and resolved on 8-16% Tris-glycine gels (Invitrogen). Proteins were transferred to 0.45 μm nitrocellulose membranes (Invitrogen) following published protocols (Towbin et al., 1979). Membranes were blocked overnight at 4^{o}C in phosphate-buffered saline (PBS, pH 7.2) with 5% (wt/vol) non-fat dried milk. Membranes were incubated with (i) 1/100 immune ascitic fluid obtained from mice inoculated with MODV (American Type Culture Collection) or a (ii) 1/100 pooled suspension of anti-WNV E protein monoclonal antibodies 3.67G and 3.91D (Millipore, Billerica, MA) for 1 hr at room temperature. Membranes were then washed and incubated with 1/2000 horseradish peroxidase-conjugated anti-mouse IgG antibody (Invitrogen) for 1 hr at room temperature. Specifically bound antibody was visualized using 3,3'-diaminobenzidine (0.05% in PBS with 0.018% H_2O_2).

Plaque assays

Viruses were subjected to serial tenfold dilutions, inoculated onto confluent monolayers of Vero cells in 35-mm culture dishes then incubated at 37°C for 60 min. Three milliliters of neutral red-deficient minimum essential medium (Invitrogen) supplemented with 2% FBS, antibiotics and 1% agar were added to each well. Plates were incubated at 37°C for 3, 5 or 7 days for WNV, chimeric virus and MODV plaque

assays, respectively. Another 3 ml of the same medium containing 0.22% neutral red was then added to each well, and plaques were counted 24 h later. Viral titers were expressed as plaque-forming units per milliliter (pfu/ml).

Plaque morphology comparisons

Viruses were inoculated onto confluent monolayers of Vero cells in 35-mm culture dishes then incubated at 37°C for 60 min. Three milliliters of neutral red-deficient minimum essential medium (Invitrogen) supplemented with 2% FBS, antibiotics and 1% agar were added to each well, and plates were incubated at 37°C for 3, 5 or 7 days. To fix the cells, 2 ml of 10% formaldehyde was added directly onto each agar overlay and the plates were incubated at 37°C for 60 min. Agar overlays were gently removed, and 0.5 ml of 0.25% crystal violet (w/v) in 20% methanol was added to each well. Once the desired intensity was reached, plates were rinsed several times with tap water and photographed.

Results

We initially attempted to create chimeric viruses by replacing the C-prM-E and prM-E genes of the MODV infectious cDNA clone with the corresponding sequences of WNV and CxFV using restriction enzyme digestion and direct cloning strategies (data not shown). More than 2,000 bacterial colonies were screened by PCR but none contained full-length C-prM-E or prM-E sequences from the heterologous virus. Approximately 10% of the colonies contained WNV or CxFV sequences that had been truncated or contained transposon insertions. These findings led us to speculate that the structural genes of WNV and CxFV are toxic to *E. coli* cells. In order to overcome this

problem, the use of bacteria and traditional cloning was replaced by a fusion PCR-based strategy coupled to an *in vitro* transcription-free system for virus production. Similar methodologies have been developed for other arboviruses (Edmonds et al., 2013; Kohl et al., 2004).

Four full-length chimeric flavivirus fusion products, designated fpMODV-WNV(C-prM-E), fpMODV-WNV(prM-E), fpMODV-CxFV(C-prM-E) and fpMODV-CxFV(prM-E), were generated by substituting the C-prM-E and prM-E genes of MODV with the corresponding regions of WNV and CxFV. The strategy used to generate fpMODV-WNV(prM-E) is shown in Figure 1, and a similar approach was used to create the three other full-length fusion products. Each full-length flavivirus fusion product as well as each intermediate reaction product was successfully observed by agarose gel electrophoresis (Figure 2). Full-length constructs were transfected into BSR-T7/5 cells. Since all of the full-length products contain a T7 promoter at the 5' end and because BSR-T7/5 cells constitutively express T7 RNA polymerase (Buchholz et al., 1999), there was no need to perform an *in vitro* transcription before the transfection. At 4 days post-transfection, aliquots of each supernatant were collected and inoculated onto Vero, BHK-21 and C6/36 cells. Supernatants were harvested from these cell cultures at 4 days p.i. (BHK-21 cells) or 7 to 9 days p.i. (Vero and C6/36 cells) then passed several more times in the same cell type (or, where specified, a different cell type). Cell monolayers and supernatants were harvested and tested for evidence of virus infection by RT-PCR, Western blot and plaque assay.

Chimeric virus was successfully generated in BSR-T7/5 cells transfected with fpMODV-WNV(prM-E). None of the other full-length chimeric flavivirus fusion products produced detectable virus under these conditions. The chimeric virus, designated MODV-WNV(prM-E), possessed the capacity to infect and replicate within vertebrate but not mosquito cells (Figures 3-5; data not shown). Supernatants harvested from MODV-WNV(prM-E)-infected Vero and BHK-21 cells produced distinct plaques in Vero cells whereas supernatants harvested from C6/36 cells inoculated with the virus did not (Figure 3; data not shown). MODV-WNV(prM-E) plaques were larger and could be visualized earlier than MODV plaques but were smaller and visualized later than WNV plaques. At 3 days p.i., MODV and MODV-WNV(prM-E) plaques were barely visible (and too small to be measured accurately) whereas WNV plaques had a mean diameter ± standard deviation of 1.9 ± 0.15mm. At 5 days p.i., MODV, MODV-WNV(prM-E) and WNV plaques were 0.1 ± 0.04, 1.8 ± 0.14 and 7.5 ± 0.46 mm in diameter, respectively. At 7 days p.i., MODV, MODV-WNV(prM-E) and WNV plaques were 0.9 ± 0.11, 2.9 ± 0.20 and 11.7 ± 0.85 mm in diameter, respectively. The plaque sizes of MODV-WNV(prM-E) differed significantly from the plaques sizes of the parental viruses at 5 and 7 days p.i. (T test, $P<0.001$).

Chimeric flavivirus RNA was detected by RT-PCR in supernatants harvested from Vero and BHK-21 cells, but not C6/36 cells, that had been inoculated with MODV-WNV(prM-E) (Figure 4). Nucleotide sequencing of the RT-PCR products confirmed these findings. WNV antigen was detected in cell lysates harvested from MODV-WNV(prM-E)-inoculated Vero cells, but not C6/36 cells, in Western blots performed

using WNV-specific monoclonal antibodies (Figure 5). MODV antigen was not detected by Western blot in any cells inoculated with chimeric virus or MODV (both fusion-PCR-derived and wild-type MODV) when commercial immune ascitic fluid obtained from mice infected with MODV was used, possibly because the mice failed to generate a sufficient immune response.

It is interesting to note that the chimeric virus did not always produce CPE in Vero cells (Table 3). CPE was not observed in Vero cells directly inoculated with supernatants harvested from fpMODV-WNV(prM-E)-transfected BSR-T7 cells. An additional passage in Vero cells also failed to result in CPE despite the detection of chimeric viral RNA in these cultures by RT-PCR. However, after a third passage in Vero cells, CPE was clearly observed. In contrast, CPE was observed after one passage in Vero cells when the chimeric virus first underwent one passage in BHK-21 cells (Figure 6). It was difficult to monitor the BHK-21 cells for the presence of virus-induced CPE because all of these cultures, including the mock-inoculated controls, displayed considerable cell death at 4 days p.i.

We sequenced the complete C-prM-E genes of chimeric virus before and after it had been subjected to multiple cell culture passages to assess the genetic stability of the virus as well as to determine whether the acquisition of mutations within the structural genes could explain why some virus stocks possessed the ability to cause CPE in Vero cells while others did not. First, the entire C-prM-E region of MODV-WNV(prM-E) harvested from transfected BSR-T7/5 cell cultures were sequenced, and shown to contain one transition (coordinate 1457) resulting in a conservative substitution when

compared to the corresponding region of the parental WNV (Table 4). We also sequenced the C-prM-E genes of chimeric virus that had undergone one passage in BHK-21 cells followed by two passages in Vero cells. Three additional transitions were identified, two mutations were silent and the other was conservative. In addition, we sequenced the C-prM-E region of chimeric virus that had undergone three passages in Vero cells and identified the change in nucleotide coordinate 1457 and four extra substitutions. One mutation was silent, one conservative and two were non-conservative.

MODV-WNV(prM-E) and MODV demonstrated similar replication kinetics and yields in Vero cells while WNV replicated faster and produced a higher peak titer (Figure 6). The chimeric virus and MODV reached mean peak titers of 7 ($\pm$ 0.06) log10 pfu/ml at 5 days p.i. and 6.7 ($\pm$ 0.05) log10 pfu/ml at 4 days p.i., respectively. In contrast, the mean peak titer for WNV was 22 to 48-fold higher and occurred 2 to 3 days earlier.

Discussion

Most chimeric flaviviruses have been developed for vaccine purposes. In these studies, live-attenuated vaccine candidates were created by inserting specific genetic elements (typically the prM-E genes) of the flavivirus of interest into a full-length infectious cDNA backbone of another flavivirus such as the YFV vaccine vector, YFV-17D, or an attenuated strain of DENV (Blaney et al., 2008; Chambers et al., 1999; Durbin et al., 2013; Guirakhoo et al., 2000; Guy et al., 2010; Lai and Monath, 2003; Wright et al., 2008). The construction and characterization of chimeric flaviviruses has also provided critical information on the genetic elements that modulate the differential

vector ranges, transmissibilities and disease phenotypes of divergent flaviviruses. Several of these studies have been performed using representative flaviviruses from the tick-borne and mosquito-borne groups (Engel et al., 2011; Engel et al., 2010; Pletnev et al., 1992; Pletnev et al., 1993; Pletnev and Men, 1998; Tumban et al., 2011). More pertinent to this investigation are the few studies that describe the construction and characterization of viral chimeras between NKV and arthropod/vertebrate flaviviruses (Charlier et al., 2010; Charlier et al., 2004; Tumban et al., 2013). Five chimeric flaviviruses have now been created between viruses from these two groups. The first chimeric virus was generated by substituting the prM-E genes of an infectious YFV cDNA infectious clone with the homologous genes of MODV (Charlier et al., 2004) and the second contains the prM-E genes of MODV in a DENV-2 backbone (Charlier et al., 2010). Both chimeric viruses replicated in C6/36 cells indicating that the inability of NKV flaviviruses to infect mosquito cells is not mediated by the viral envelope but by a post-entry event. Two more chimeric viruses were constructed by replacing the conserved pentanucleotide sequence (CPS) or variable region (VR) of the 3' UTR of a DENV-4 infectious clone with the corresponding region of MODV. Both viruses could infect C6/36 cells and adult mosquitoes at similar efficiencies to DENV-4 suggesting that the CPS and VR of mosquito/vertebrate flaviviruses are not required for mosquito infectivity. We too have successfully created a chimeric virus using a NKV and mosquito-borne flavivirus but, unlike the above studies, our virus was constructed using the vertebrate-specific virus as the backbone. The virus, designated MODV-WNV(prM-E), was created by replacing the prM-E genes of a MODV infectious clone with the

corresponding sequences of WNV. MODV-WNV(prM-E) possesses the capacity to infect and replicate within vertebrate but not mosquito cell cultures indicating that there are sequence elements outside of the prM-E region that dictate the vertebrate-specific host range of MODV.

The fusion product designated fpMODV-CxFV(prM-E), which was created by replacing the prM and E genes of MODV with the homologous sequences of CxFV, failed to yield detectable virus. This finding is in contrast to the numerous studies that report the successful production of chimeric virus after the prM-E genes of one flavivirus are replaced with those of another (Arroyo et al., 2004; Butrapet et al., 2002; Huang et al., 2003; Maharaj et al., 2012; Pletnev et al., 2001; Pletnev et al., 1993; Pletnev et al., 2002; Pugachev et al., 2004). However, all of these studies were performed with flaviviruses that possess at least one common host. Indeed, although chimeric viruses have been created between viruses as divergent as tick- and mosquito-borne flaviviruses, and NKV and mosquito-borne flaviviruses, all viruses within these groups possess the ability to replicate within vertebrate cells. In contrast, ISFs and NKV flaviviruses do not possess a common host by virtue of their insect and vertebrate-specific phenotypes. Thus, the generation of chimeric viruses between ISFs and NKV flaviviruses may not be achievable or, at the very least, will prove extremely challenging because their genomes and resulting translation products may be fundamentally incompatible as a consequence of their evolutionary divergence and specialization to vastly different hosts.

Conserved complementary cyclization sequences reside within the capsid gene and 3’ UTR of the flavivirus genome. These sequences interact with one another to facilitate genome cyclization and are essential for viral replication (Khromykh et al., 2001; Kofler et al., 2006). Thus, one explanation for the inability to produce infectious virus with the fusion products containing the C-prM-E genes of WNV and CxFV is because the genome cyclization elements within the 3’ UTR of MODV and the C gene of the alternate virus do not have sufficient complementary to support genome cyclization. In this regard, replacement of the 3’UTR of a DENV-4 infectious clone with the corresponding region of MODV also failed to produce virus (Tumban et al., 2013). Virus was also unable to be recovered when both UTRs and the C gene of DENV-4 or LGTV were replaced with those of MODV, despite the presence of compatible cyclization elements (Tumban et al., 2013; Tumban et al. 2011). The authors speculated that infectious virus was not producted because fundamental incompatibilities exist between the UTRs and replication complexes of highly divergent (e.g. mosquito-borne, tick-borne and vertebrate-specific) flaviviruses. However, C-prM-E gene substitutions between divergent flaviviruses have occasionally proven successful; Pletnev and colleagues produced chimeric virus after replacing all three structural genes of DENV-4 with those of TBEV (Pletnev et al., 1992).

The inability to produce chimeric virus with fpMODV-CxFV(prM-E), fpMODV-CxFV(C-prM-E) and fpMODV-WNV(C-prM-E) is unlikely due to aberrant replication complex formation. Assembly of the viral replication complex should not have been impeded due to mismatches between the various viral and cellular proteins that interact

during this process because no nonstructural gene substitutions were made. It is also unlikely that correct proteolytic processing of the chimeric polyproteins could not occur. Amino acid sequence alignments have shown that the predicted cleavage sites required for proteolytic cleavage of the CxFV and MODV polyproteins are similar to one another and to those of WNV and other dual-host flaviviruses (Castle et al., 1986; Castle et al., 1985; Hoshino et al., 2007; Leyssen et al., 2002). Although the junctions of all four constructs were sequenced and shown to contain no nucleotide errors, these constructs were not sequenced in their entirety and thus, we cannot dismiss the possibility that the non-viable constructs contained lethal mutations outside the junctions that occurred during one of the PCR amplifications.

The replication kinetics and yields of MODV-WNV(prM-E) in Vero cells where similar to those of MODV. These data suggest that genetic elements outside of the prM-E region dictate the *in vitro* replication profiles of NKV flaviviruses in vertebrate cells. Other studies have also shown that chimeric flaviviruses generated by prM-E gene substitutions exhibit replication kinetics and yields similar to the virus from which the nonstructural genes were derived but distinct from the virus that contributed the prM-E sequences (Charlier et al., 2004; Huang et al., 2005; Maharaj et al., 2012). For instance, the *in vitro* replication kinetics of a chimeric virus that possessed the prM-E genes of MODV in a YFV-17D backbone were similar to those of YFV-17D but distinct from MODV which reached a higher peak titer (Charlier et al., 2004). Although the chimeric virus and MODV displayed similar *in vitro* replication kinetics, these two viruses exhibited differential plaque morphologies in Vero cells. MODV-WNV(prM-E) plaques

were at least threefold larger than MODV plaques but approximately fourfold smaller than WNV plaques. These findings indicate that genetic elements both within and outside of the prM-E region modulate the plaque sizes of NKV flaviviruses.

MODV-WNV(prM-E) did not always cause CPE in Vero cells, and the occurrence of CPE appeared dependent on the passage history of the virus. MODV-WNV(prM-E) was able to induce CPE after a single passage in Vero cells if it had first been cultured in BHK-21 cells. In contrast, CPE did not occur in Vero cells until the third passage when the virus had not been passaged in BHK-21 cells. One explanation for these findings is that MODV-WNV(prM-E) is better acclimated for growth in BHK-21 cells. Alternatively, repeated passaging of the virus in Vero cells could have resulted in the accumulation of mutations that altered its ability to induce CPE in this cell type. In this regard, the envelope gene sequence of chimeric virus derived from the original inoculum contained one non-synonymous mutation when compared to the corresponding region of parental virus while chimeric viruses had undergone three passages in BHK-21 and/or Vero cells acquired two to four additional mutations in this region. Whether these mutations, or mutations that may have occurred elsewhere in the viral genome, altered the ability of the virus to induce CPE is not known but it does offer a likely explanation.

Fusion-PCR has been used for the successful generation of full-length infectious flavivirus cDNA clones and chimeric flaviviruses (Caufour et al., 2001; Charlier et al., 2003; Edmonds et al., 2013; Gritsun and Gould, 1995; Mathenge et al., 2004). Fusion-PCR has several advantages over conventional cloning. First, the latter approach requires the presence of unique restriction enzyme sites at the cloning site which are often not

present and therefore need to be engineered into the vector. Additionally, restriction enzyme digestions and ligations are expensive and time-consuming. Another advantage of the fusion-PCR technique is that it does not require the use of bacteria when a promoter is present in the resulting amplions. This is important because mutations can arise during plasmid amplification in bacterial cells. Moreover, the genes of interest may be toxic to bacteria and thus, it may be difficult or impossible to propagate bacteria possessing plasmids with certain sequence elements. The intrinsic toxicity of full-length flavivirus cDNAs in bacterial cells has been well documented (Pu et al., 2011; Ruggli and Rice, 1999) and is a likely explanation for our inability to create MODV-WNV and MODV-CxFV chimeras using restriction enzyme digestion and direct cloning (methods and data not shown). We complement the fusion PCR-based system by using cells that stably express T7 RNA polymerase for the transfections, thereby further streamlining the process by eliminating the need to perform *in vitro* transcriptions. Another bacterium-free approach that does not require the *in vitro* synthesis of viral RNAs has also been described (Edmonds et al., 2013). This highly innovative method, which is based on a circular polymerase extension cloning reaction performed with Phusion DNA polymerase, was recently used to generate full-length infectious WNV cDNA.

In summary, we report the first chimeric flavivirus to be constructed using a NKV flavivirus infectious cDNA clone as the backbone. The chimeric virus, which contains the prM-E genes of WNV, could replicate within vertebrate but not mosquito cells indicating that sequence elements outside of the prM-E region preclude NKV flavivirus replication in mosquito cells. We also report the first attempts to create a

chimeric flavivirus between an ISF and NKV flavivirus. Two constructs were generated, including one that contains the CxFV prM-E genes in a MODV backbone, but neither yielded detectable virus. Most success in the generation of chimeric flaviviruses has been achieved through prM-E gene substitutions. However, unlike our study, all previous studies were performed using flaviviruses that share a common host. These findings indicate that the successful generation of chimeric viruses between ISFs and NKV flaviviruses will prove extremely challenging due to the evolutionary divergence and differential host ranges of these viruses.

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References

Arroyo, J., Miller, C., Catalan, J., Myers, G.A., Ratterree, M.S., Trent, D.W., Monath, T.P., 2004. ChimeriVax-West Nile virus live-attenuated vaccine: preclinical evaluation of safety, immunogenicity, and efficacy. Journal of virology 78, 12497-12507.

Bell, J.F., Thomas, L.A., 1964. A New Virus, "Mml", Enzootic in Bats (Myotis Lucifugus) of Montana. The American journal of tropical medicine and hygiene 13, 607-612.

Blaney, J.E., Jr., Speicher, J., Hanson, C.T., Sathe, N.S., Whitehead, S.S., Murphy, B.R., Pletnev, A.G., 2008. Evaluation of St. Louis encephalitis virus/dengue virus type

4 antigenic chimeric viruses in mice and rhesus monkeys. Vaccine 26, 4150-4159.

Blitvich, B., Lin, M, Dorman, KS, Soto, V, Hovav E, Tucker, BJ, Staley, M, Platt, KB, Bartholomay, LC., 2009. Genomic sequence and phylogenetic analysis of Culex flavivirus, an insect-specific flavivirus, isolated from *Culex pipiens* in Iowa (Diptera: Culicidae). J Med Entomol In press.

Buchholz, U.J., Finke, S., Conzelmann, K.-K., 1999. Generation of Bovine Respiratory Syncytial Virus (BRSV) from cDNA: BRSV NS2 Is Not Essential for Virus Replication in Tissue Culture, and the Human RSV Leader Region Acts as a Functional BRSV Genome Promoter. Journal of Virology 73, 251-259.

Burns, K.F., Farinacci, C.J., 1956. Virus of bats antigenically related to St. Louis encephalitis. Science 123, 227.

Butrapet, S., Rabablert, J., Angsubhakorn, S., Wiriyarat, W., Huang, C., Kinney, R., Punyim, S., Bhamarapravati, N., 2002. Chimeric dengue type 2/type 1 viruses induce immune responses in cynomolgus monkeys. Southeast Asian J Trop Med Public Health 33, 589-599.

Castle, E., Leidner, U., Nowak, T., Wengler, G., Wengler, G., 1986. Primary structure of the West Nile flavivirus genome region coding for all nonstructural proteins. Virology 149, 10-26.

Castle, E., Nowak, T., Leidner, U., Wengler, G., Wengler, G., 1985. Sequence analysis of the viral core protein and the membrane-associated proteins V1 and NV2 of the flavivirus West Nile virus and of the genome sequence for these proteins. Virology 145, 227-236.

Caufour, P.S., Motta, M.C., Yamamura, A.M., Vazquez, S., Ferreira, II, Jabor, A.V., Bonaldo, M.C., Freire, M.S., Galler, R., 2001. Construction, characterization and immunogenicity of recombinant yellow fever 17D-dengue type 2 viruses. Virus Res 79, 1-14.

Chambers, T.J., Nestorowicz, A., Mason, P.W., Rice, C.M., 1999. Yellow fever/Japanese encephalitis chimeric viruses: construction and biological properties. Journal of virology 73, 3095-3101.

Charlier, N., Davidson, A., Dallmeier, K., Molenkamp, R., De Clercq, E., Neyts, J., 2010. Replication of not-known-vector flaviviruses in mosquito cells is restricted by intracellular host factors rather than by the viral envelope proteins. The Journal of general virology 91, 1693-1697.

Charlier, N., Molenkamp, R., Leyssen, P., Paeshuyse, J., Drosten, C., Panning, M., De Clercq, E., Bredenbeek, P.J., Neyts, J., 2004. Exchanging the yellow fever virus envelope proteins with Modoc virus prM and E proteins results in a chimeric virus that is neuroinvasive in SCID mice. Journal of virology 78, 7418-7426.

Charlier, N., Molenkamp, R., Leyssen, P., Vandamme, A.M., De Clercq, E., Bredenbeek, P., Neyts, J., 2003. A rapid and convenient variant of fusion-PCR to construct chimeric flaviviruses. J Virol Methods 108, 67-74.

Chen, Y., Maguire, T., Marks, R.M., 1996. Demonstration of binding of dengue virus envelope protein to target cells. Journal of virology 70, 8765-8772.

Constantine, D.G., Woodall, D.F., 1964. Latent Infection of Rio Bravo Virus in Salivary Glands of Bats. Public Health Rep 79, 1033-1039.

Cook, S., Moureau, G., Kitchen, A., Gould, E.A., de Lamballerie, X., Holmes, E.C., Harbach, R.E., 2012. Molecular evolution of the insect-specific flaviviruses. The Journal of general virology 93, 223-234.

Crabtree, M.B., Sang, R.C., Stollar, V., Dunster, L.M., Miller, B.R., 2003. Genetic and phenotypic characterization of the newly described insect flavivirus, Kamiti River virus. Arch Virol 148, 1095-1118.

Durbin, A.P., Wright, P.F., Cox, A., Kagucia, W., Elwood, D., Henderson, S., Wanionek, K., Speicher, J., Whitehead, S.S., Pletnev, A.G., 2013. The live attenuated chimeric vaccine rWN/DEN4Delta30 is well-tolerated and immunogenic in healthy flavivirus-naive adult volunteers. Vaccine 31, 5772-5777.

Edmonds, J., van Grinsven, E., Prow, N., Bosco-Lauth, A., Brault, A.C., Bowen, R.A., Hall, R.A., Khromykh, A.A., 2013. A novel bacterium-free method for generation of flavivirus infectious DNA by circular polymerase extension reaction allows accurate recapitulation of viral heterogeneity. Journal of virology 87, 2367-2372.

Egloff, M.P., Benarroch, D., Selisko, B., Romette, J.L., Canard, B., 2002. An RNA cap (nucleoside-2'-O-)-methyltransferase in the flavivirus RNA polymerase NS5: crystal structure and functional characterization. The EMBO journal 21, 2757-2768.

Engel, A.R., Mitzel, D.N., Hanson, C.T., Wolfinbarger, J.B., Bloom, M.E., Pletnev, A.G., 2011. Chimeric tick-borne encephalitis/dengue virus is attenuated in *Ixodes scapularis* ticks and *Aedes aegypti* mosquitoes. Vector borne and zoonotic diseases 11, 665-674.

Engel, A.R., Rumyantsev, A.A., Maximova, O.A., Speicher, J.M., Heiss, B., Murphy, B.R., Pletnev, A.G., 2010. The neurovirulence and neuroinvasiveness of chimeric tick-borne encephalitis/dengue virus can be attenuated by introducing defined mutations into the envelope and NS5 protein genes and the 3 ' non-coding region of the genome. Virology 405, 243-252.

Falgout, B., Pethel, M., Zhang, Y.M., Lai, C.J., 1991. Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. Journal of virology 65, 2467-2475.

Gould, E.A., de Lamballerie, X., Zanotto, P.M., Holmes, E.C., 2003. Origins, evolution, and vector/host coadaptations within the genus Flavivirus. Advances in virus research 59, 277-314.

Gritsun, T.S., Gould, E.A., 1995. Infectious transcripts of tick-borne encephalitis virus, generated in days by RT-PCR. Virology 214, 611-618.

Gubler, D.J., Kuno, G., Markoff, L., 2007. Flaviviruses. In Fields Virology. Fifth Edition. Editors: Knipe, D.M., Howley, P.M. pp. 1153-1252. Lippincott Williams and Wilkins. Philadelphia, PA.

Guirakhoo, F., Bolin, R.A., Roehrig, J.T., 1992. The Murray Valley encephalitis virus prM protein confers acid resistance to virus particles and alters the expression of epitopes within the R2 domain of E glycoprotein. Virology 191, 921-931.

Guirakhoo, F., Weltzin, R., Chambers, T.J., Zhang, Z.X., Soike, K., Ratterree, M., Arroyo, J., Georgakopoulos, K., Catalan, J., Monath, T.P., 2000. Recombinant chimeric yellow fever-dengue type 2 virus is immunogenic and protective in nonhuman primates. Journal of virology 74, 5477-5485.

Guy, B., Guirakhoo, F., Barban, V., Higgs, S., Monath, T.P., Lang, J., 2010. Preclinical and clinical development of YFV 17D-based chimeric vaccines against dengue, West Nile and Japanese encephalitis viruses. Vaccine 28, 632-649.

Haddow, A.D., Guzman, H., Popov, V.L., Wood, T.G., Widen, S.G., Haddow, A.D., Tesh, R.B., Weaver, S.C., 2013. First isolation of Aedes flavivirus in the Western Hemisphere and evidence of vertical transmission in the mosquito *Aedes (Stegomyia) albopictus* (Diptera: Culicidae). Virology 440, 134-139.

Heinz, F.X., Stiasny, K., Puschner-Auer, G., Holzmann, H., Allison, S.L., Mandl, C.W., Kunz, C., 1994. Structural changes and functional control of the tick-borne encephalitis virus glycoprotein E by the heterodimeric association with protein prM. Virology 198, 109-117.

Hoshino, K., Isawa, H., Tsuda, Y., Yano, K., Sasaki, T., Yuda, M., Takasaki, T., Kobayashi, M., Sawabe, K., 2007. Genetic characterization of a new insect flavivirus isolated from *Culex pipiens* mosquito in Japan. Virology 359, 405-414.

Huang, C.Y., Butrapet, S., Tsuchiya, K.R., Bhamarapravati, N., Gubler, D.J., Kinney, R.M., 2003. Dengue 2 PDK-53 virus as a chimeric carrier for tetravalent dengue vaccine development. Journal of virology 77, 11436-11447.

Huang, C.Y., Silengo, S.J., Whiteman, M.C., Kinney, R.M., 2005. Chimeric dengue 2 PDK-53/West Nile NY99 viruses retain the phenotypic attenuation markers of the candidate PDK-53 vaccine virus and protect mice against lethal challenge with West Nile virus. Journal of virology 79, 7300-7310.

Johnson, H.N., 1967. Ecological implications of antigenically related mammalian viruses for which arthropod vectors are unknown and avian associated soft tick viruses. Jpn J Med Sci Biol 20 Suppl, 160-166.

Khromykh, A.A., Meka, H., Guyatt, K.J., Westaway, E.G., 2001. Essential role of cyclization sequences in flavivirus RNA replication. Journal of virology 75, 6719-6728.

Kofler, R.M., Hoenninger, V.M., Thurner, C., Mandl, C.W., 2006. Functional analysis of the tick-borne encephalitis virus cyclization elements indicates major differences between mosquito-borne and tick-borne flaviviruses. Journal of virology 80, 4099-4113.

Kohl, A., Hart, T.J., Noonan, C., Royall, E., Roberts, L.O., Elliott, R.M., 2004. A bunyamwera virus minireplicon system in mosquito cells. Journal of virology 78, 5679-5685.

Kuno, G., 2007. Host range specificity of flaviviruses: correlation with in vitro replication. Journal of medical entomology 44, 93-101.

Kuno, G., Chang, G.J., Tsuchiya, K.R., Karabatsos, N., Cropp, C.B., 1998. Phylogeny of the genus Flavivirus. Journal of virology 72, 73-83.

Lai, C.J., Monath, T.P., 2003. Chimeric flaviviruses: novel vaccines against dengue fever, tick-borne encephalitis, and Japanese encephalitis. Advances in virus research 61, 469-509.

Leyssen, P., Charlier, N., Lemey, P., Billoir, F., Vandamme, A.M., De Clercq, E., de Lamballerie, X., Neyts, J., 2002. Complete genome sequence, taxonomic assignment, and comparative analysis of the untranslated regions of the Modoc virus, a flavivirus with no known vector. Virology 293, 125-140.

Lindenbach, B.D., Thiel, H.-J., Rice, C.M., 2007. *Flaviviridae*: the viruses and their replication. In Fields Virology. Fifth Edition. Editors: Knipe, D.M. Howley, P.M. pp. 1101-1152. Lippincott Williams and Wilkins. Philadelphia, PA.

Maharaj, P.D., Anishchenko, M., Langevin, S.A., Fang, Y., Reisen, W.K., Brault, A.C., 2012. Structural gene (prME) chimeras of St Louis encephalitis virus and West Nile virus exhibit altered in vitro cytopathic and growth phenotypes. The Journal of general virology 93, 39-49.

Markoff, L., 2003. 5'- and 3'-noncoding regions in flavivirus RNA. Advances in virus research 59, 177-228.

Mathenge, E.G., Parquet Mdel, C., Funakoshi, Y., Houhara, S., Wong, P.F., Ichinose, A., Hasebe, F., Inoue, S., Morita, K., 2004. Fusion PCR generated Japanese encephalitis virus/dengue 4 virus chimera exhibits lack of neuroinvasiveness, attenuated neurovirulence, and a dual-flavi immune response in mice. The Journal of general virology 85, 2503-2513.

Pletnev, A.G., Bray, M., Hanley, K.A., Speicher, J., Elkins, R., 2001. Tick-borne Langat/mosquito-borne dengue flavivirus chimera, a candidate live attenuated vaccine for protection against disease caused by members of the tick-borne encephalitis virus complex: evaluation in rhesus monkeys and in mosquitoes. Journal of virology 75, 8259-8267.

Pletnev, A.G., Bray, M., Huggins, J., Lai, C.J., 1992. Construction and characterization of chimeric tick-borne encephalitis/dengue type 4 viruses. Proceedings of the National Academy of Sciences of the United States of America 89, 10532-10536.

Pletnev, A.G., Bray, M., Lai, C.J., 1993. Chimeric tick-borne encephalitis and dengue type 4 viruses: effects of mutations on neurovirulence in mice. Journal of virology 67, 4956-4963.

Pletnev, A.G., Men, R., 1998. Attenuation of the Langat tick-borne flavivirus by chimerization with mosquito-borne flavivirus dengue type 4. Proceedings of the National Academy of Sciences of the United States of America 95, 1746-1751.

Pletnev, A.G., Putnak, R., Speicher, J., Wagar, E.J., Vaughn, D.W., 2002. West Nile virus/dengue type 4 virus chimeras that are reduced in neurovirulence and peripheral virulence without loss of immunogenicity or protective efficacy. Proceedings of the National Academy of Sciences of the United States of America 99, 3036-3041.

Pu, S.Y., Wu, R.H., Yang, C.C., Jao, T.M., Tsai, M.H., Wang, J.C., Lin, H.M., Chao, Y.S., Yueh, A., 2011. Successful propagation of flavivirus infectious cDNAs by

a novel method to reduce the cryptic bacterial promoter activity of virus genomes. Journal of virology 85, 2927-2941.

Pugachev, K.V., Guirakhoo, F., Mitchell, F., Ocran, S.W., Parsons, M., Johnson, B.W., Kosoy, O.L., Lanciotti, R.S., Roehrig, J.T., Trent, D.W., Monath, T.P., 2004. Construction of yellow fever/St. Louis encephalitis chimeric virus and the use of chimeras as a diagnostic tool. The American journal of tropical medicine and hygiene 71, 639-645.

Rey, F.A., Heinz, F.X., Mandl, C., Kunz, C., Harrison, S.C., 1995. The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. Nature 375, 291-298.

Rice, C.M., Lenches, E.M., Eddy, S.R., Shin, S.J., Sheets, R.L., Strauss, J.H., 1985. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. Science 229, 726-733.

Ruggli, N., Rice, C.M., 1999. Functional cDNA clones of the *Flaviviridae*: strategies and applications. Advances in virus research 53, 183-207.

Saiyasombat, R., Bolling, B.G., Brault, A.C., Bartholomay, L.C., Blitvich, B.J., 2011. Evidence of efficient transovarial transmission of Culex flavivirus by *Culex pipiens* (Diptera: Culicidae). Journal of medical entomology 48, 1031-1038.

Stadler, K., Allison, S.L., Schalich, J., Heinz, F.X., 1997. Proteolytic activation of tick-borne encephalitis virus by furin. Journal of virology 71, 8475-8481.

Stollar, V., Thomas, V.L., 1975. An agent in the *Aedes aegypti* cell line (Peleg) which causes fusion of *Aedes albopictus* cells. Virology 64, 367-377.

Tan, B.H., Fu, J., Sugrue, R.J., Yap, E.H., Chan, Y.C., Tan, Y.H., 1996. Recombinant dengue type 1 virus NS5 protein expressed in *Escherichia coli* exhibits RNA-dependent RNA polymerase activity. Virology 216, 317-325.

Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of the National Academy of Sciences of the United States of America 76, 4350-4354.

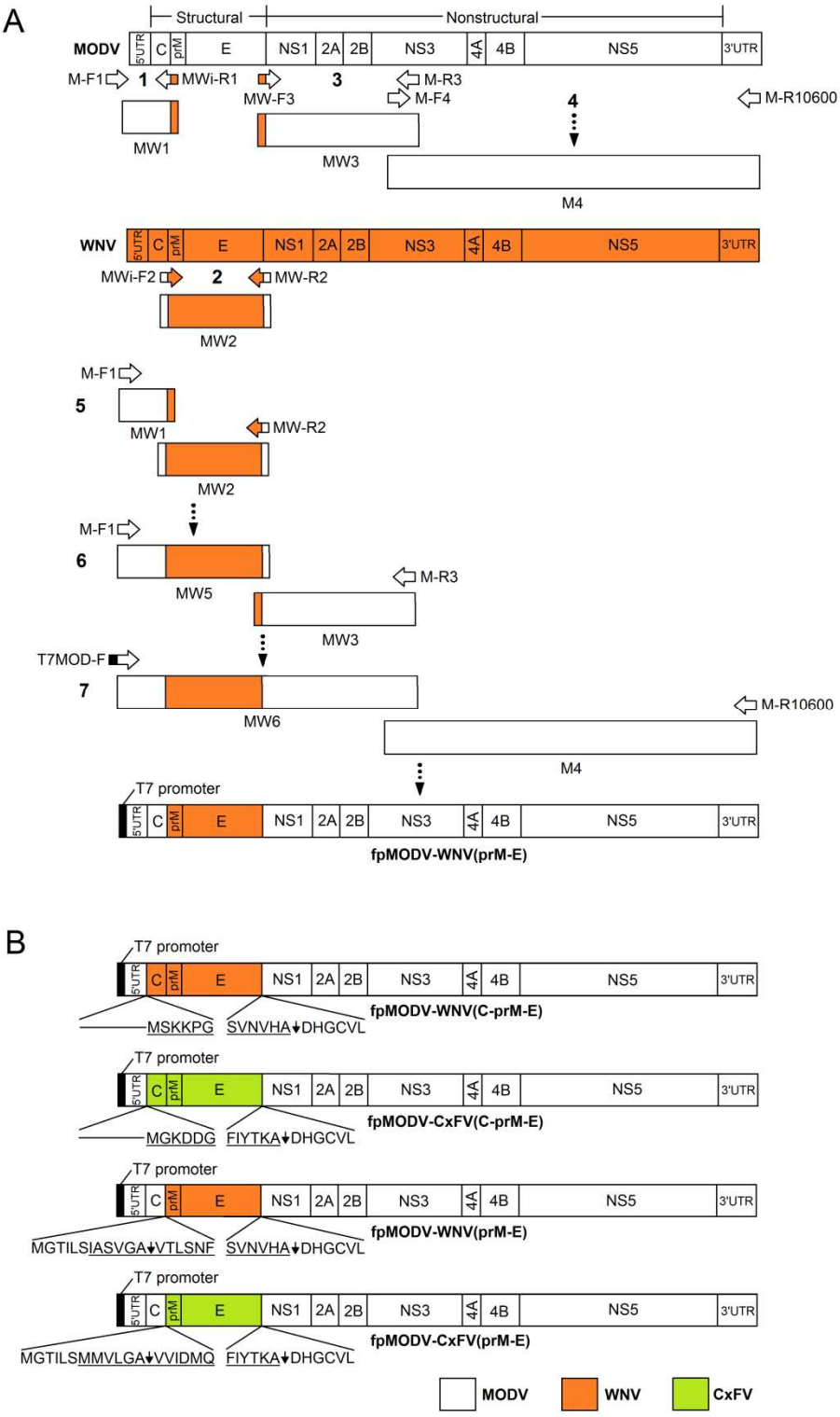
Tumban, E., Maes, N.E., Schirtzinger, E.E., Young, K.I., Hanson, C.T., Whitehead, S.S., Hanley, K.A., 2013. Replacement of conserved or variable sequences of the mosquito-borne dengue virus 3' UTR with homologous sequences from Modoc virus does not change infectivity for mosquitoes. The Journal of general virology 94, 783-788.

Tumban, E., Mitzel, D.N., Maes, N.E., Hanson, C.T., Whitehead, S.S., Hanley, K.A., 2011. Replacement of the 3' untranslated variable region of mosquito-borne dengue virus with that of tick-borne Langat virus does not alter vector specificity. The Journal of general virology 92, 841-848.

Wright, P.F., Ankrah, S., Henderson, S.E., Durbin, A.P., Speicher, J., Whitehead, S.S., Murphy, B.R., Pletnev, A.G., 2008. Evaluation of the Langat/dengue 4 chimeric virus as a live attenuated tick-borne encephalitis vaccine for safety and immunogenicity in healthy adult volunteers. Vaccine 26, 882-890.

Zarnke, R.L., Yuill, T.M., 1985. Modoc-like virus isolated from wild deer mice (*Peromyscus maniculatus*) in Alberta. Journal of wildlife diseases 21, 94-99.

Figure 1. Schematic of the fusion-PCR strategy used to generate viral chimeras.



(A). Strategy used to generate fpMODV-WNV(prM-E) in seven steps. The approximate location of primers and intermediate PCR products are shown on each viral genome (not scaled). Note that just viral sequences are depicted, the actual MODV template was pACNR-FLMODV while WNV template was viral cDNA (see materials and methods). All intermediate products and primers are further described in accompanying Table 1. Chimeric primers are represented by bicolor arrows. Steps 1-4: Products MW1, MW2, MW3 and M4 were generated by PCR with the indicated primers. These fragments were used as construction blocks in subsequent steps in fusion PCRs. Step 5: Products MW1 and MW2 were fused amplifying with primers M-F1 and MW-R2 to generate product MW5. Step 6: MW5 was fused with MW3 using primers M-F1 and M-R3 to give MW6. Step 7: In the final reaction, a full-length chimeric product was generated by fusing MW6 to M4 using primers T7-MOD-F and M-R10600. (B). Maps of final constructs highlighting the resulting amino acid chimeric sequences. Arrows indicate protease cleavage sites. Sequences from the heterologous viruses (WNV or CxFV) are underlined.

Figure 2. Amplicons generated during the construction of full-length chimeric flavivirus fusion products. The construction of each full-length chimeric flavivirus fusion product required (A-D) four conventional PCRs and (E-G) three fusion-PCRs. Amplicons are named as described in Table 2 and were visualized after 0.8% agarose gel electrophoresis and ethidium bromide staining. In Panel G, lanes 1 to 5 contain fpMODV-WNV(C-prM-E), fpMODV-CxFV(C-prM-E), full-length MODV genomic DNA, fpMODV-WNV(prM-E) and fpMODV-CxFV(prM-E), respectively. DNA ladder was included on each gel with the sizes of selected bands shown.

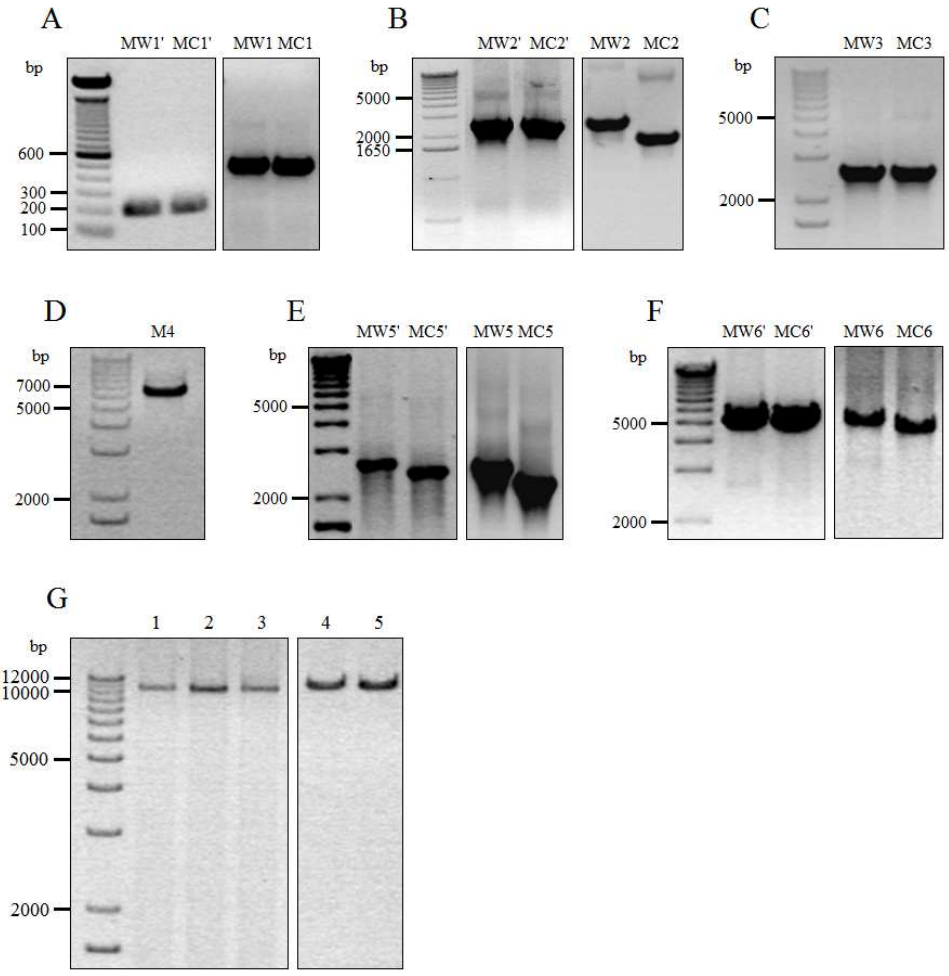


Figure 3. Comparison of the plaque morphologies of MODV-WNV(prM-E) and the parental viruses in Vero cells. Confluent monolayers of Vero cells in six-well plates were inoculated with MODV-WNV(prM-E), MODV or WNV. Cells were fixed and plaques were visualized by staining with crystal violet at 3, 5 and 7 days p.i. Images were transferred into Microsoft Photoshop and plaque diameters were measured. The chimeric virus had been passaged one in BHK-21 cells and twice in Vero cells prior to this experiment.

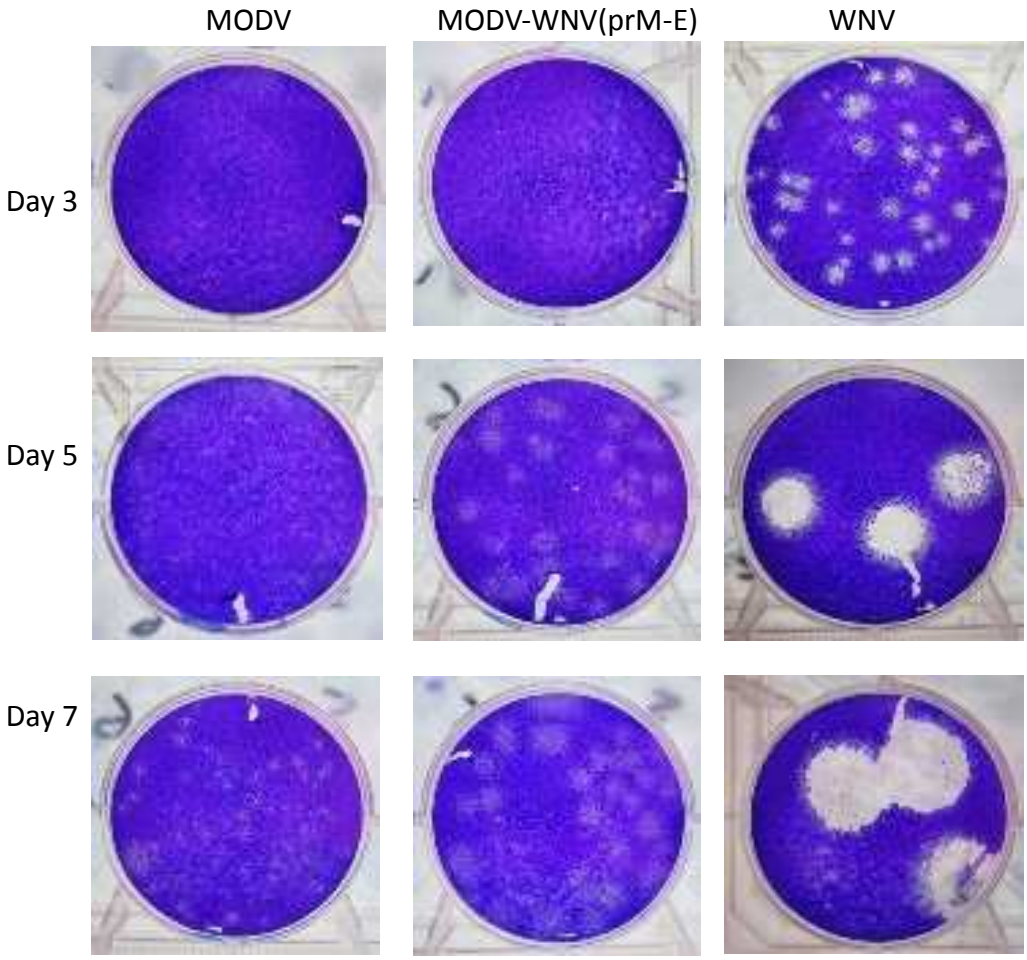
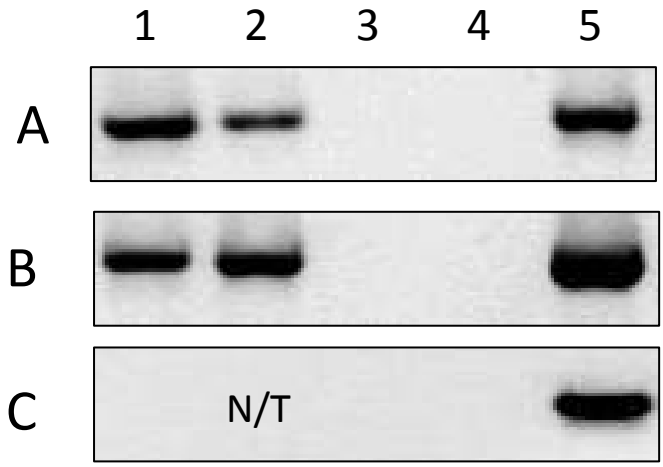


Figure 4.



Detection of chimeric viral RNA by RT-PCR in mammalian but not mosquitoes cells inoculated with MODV-WNV(prM-E). Supernatants harvested from fpMODV-WNV(prM-E)-transfected BSR-T7 cells underwent (A) three passages in Vero cells, (B) one passage in BHK-21 cells followed by two passages in Vero cells or (C) three passages in C6/36 cells. After the final passage, total RNA was extracted from culture supernatants and assayed by RT-PCR using a forward primer specific to the MODV capsid gene and a reverse primer specific to the WNV prM gene (lanes 1-2). Mock-inoculated cultures were also tested in these experiments (lane 3). Samples were treated with DNase I prior to RT-PCR (lane 2) or were left untreated (lanes 1 and 3). Negative control RT-PCRs (in which dH_2O was used as template) and positive control RT-PCRs (in which fpMODV-WNV(prM-E) was used as template) were included (lanes 4 and 5, respectively). N/T denotes not tested.

Figure 5.

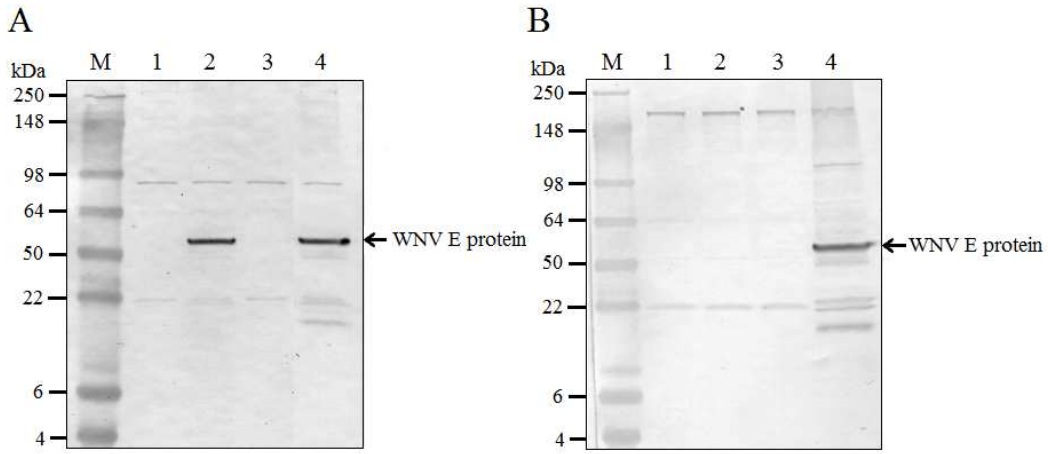
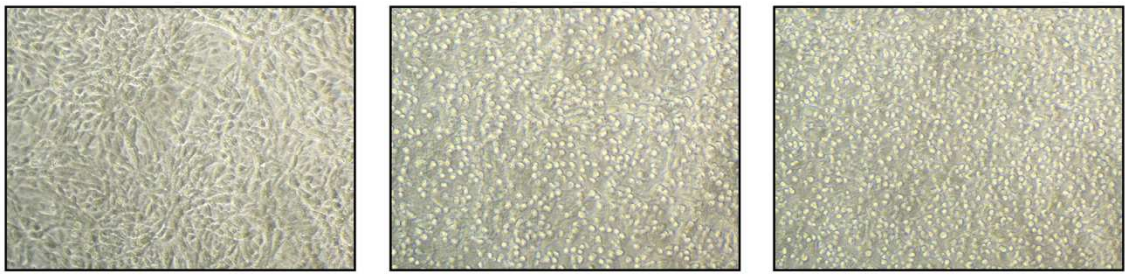


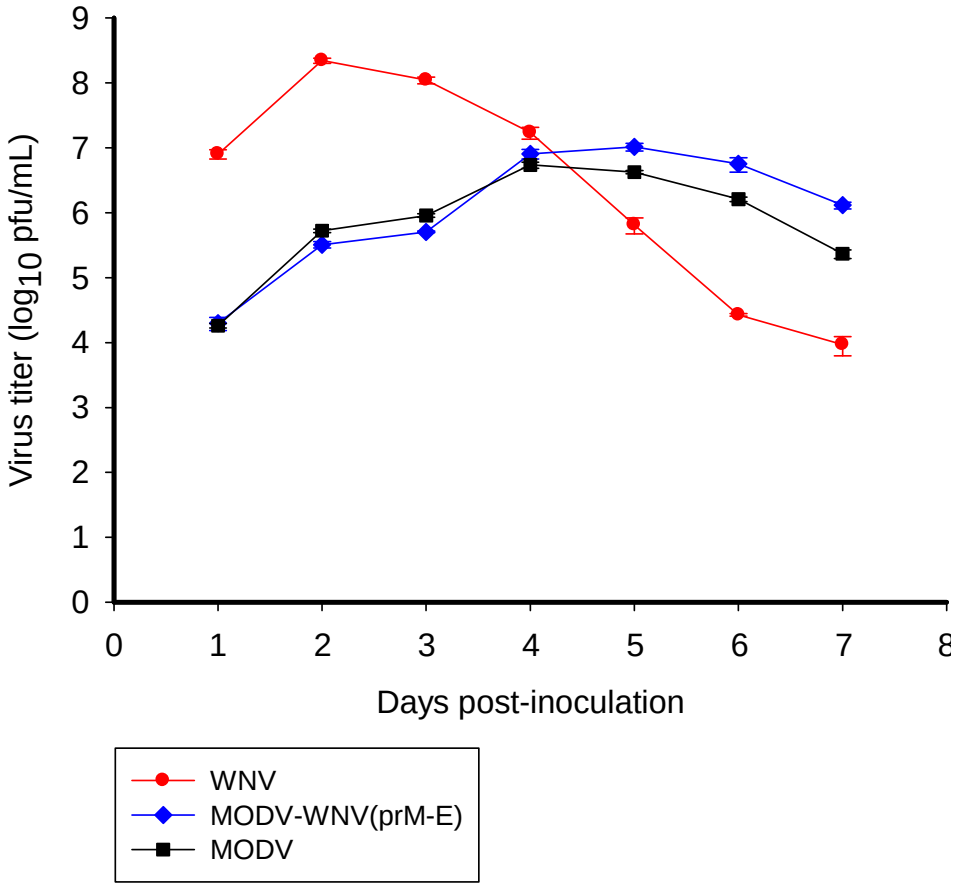
Figure 5. Western blot analysis reveals the presence of WNV antigen in Vero cells, but not C6/36 cells, inoculated with MODV-WNV(prM-E). Lysates were prepared from (A) Vero and (B) C6/36 cells that had been mock-inoculated (lane 1) or inoculated with chimeric virus (lane 2), MODV (lane 3) or WNV (lane 4) at a m.o.i. of 0.1. Lysates were harvested at 7 days p.i. and equal amounts of protein were resolved on 8-16% Tris-glycine gels and immunoblotted using a pooled suspension of anti-WNV E protein monoclonal antibodies. M denotes the SDS PAGE low-range molecular weight standards (Invitrogen). The arrow shows the expected migration position of the WNV E protein (molecular weight: 53 KDa).

Figure 6.



Detection of cytopathic effect in Vero cells inoculated with MODV-WNV(prM-E). MODV-WNV(prM-E) and MODV that had been passaged twice in Vero cells were inoculated onto fresh monolayers of Vero cells and monitored for 5 or 8 days, respectively. Mock-infected Vero cells that were incubated for 5 days were also included. Magnification = 100x

Figure 7.



Comparison of the replication kinetics of MODV-WNV(prM-E), MODV and WNV in Vero cells. Subconfluent monolayers of Vero cells were inoculated with MODV-WNV(prM-E), MODV and WNV at a m.o.i of 0.1. Supernatants were collected daily for 7 days and tested in triplicate by plaque assay. Three independent experiments were performed. Within each experiment, six replicates of each virus/dilution/timepoint were tested. Data were used to calculate mean viral titers ± standard deviation. MODV-WNV(prM-E) had been passaged one in BHK-21 cells and once in Vero cells prior to the experiments.

Table 1. PCR products generated during the construction of full-length flavivirus chimeric DNAs

Reaction No.	Reaction Type	Primers (Forward, Reverse)	PCR product	
			Name	Size (bp)
1a	PCR	M-F1, MW-R1	MW1’	191
1b		M-F1, MC-R1	MC1’	194
1c		M-F1, MWi-R1	MW1	523
1d		M-F1, MCi-R1	MC1	521
2a	RT-PCR	MW-F2, MW-R2	MW2’	2,415
2b		MC-F2, MC-R2	MC2’	2,167
2c		MWi-F2, MWR2	MW2	2,066
2d		MCi-F2, MCR2	MC2	1,777
3a,c	PCR	MW-F3, M-R3	MW3	2,575
3b,d		MC-F3, M-R3	MC3	2,580
4a-d	PCR	M-F4, M-R10600	M4	6,227
5a	Fusion-PCR	M-F1, MW-R2	MW5’	2,563
5b		M-F1, MC-R2	MC5’	2,320
5c		M-F1, MW-R2	MW5	2,542
5d		M-F1, MC-R2	MC5	2,251
6a	Fusion-PCR	M-F1, M-R3	MW6’	5,100
6b		M-F1, M-R3	MC6’	4,854
6c		M-F1, M-R3	MW6	5,079
6d		M-F1, M-R3	MC6	4,785
7a	Fusion-PCR	T7MOD-F, M-R10600	fpMODV-WNV(C-prM-E)	10,730
7b		T7MOD-F, M-R10600	fpMODV-WNV(prM-E)	10,708
7c		T7MOD-F, M-R10600	fpMODV-CxFV(C-prM-E)	10,484
7d		T7MOD-F, M-R10600	fpMODV-CxFV(prM-E)	10,415

Reactions ending with a, b, c and d were used to generate fpMODV-WNV(C-prM-E), fpMODV-CxFV(C-prM-E), fpMODV-WNV(prM-E) and fpMODV-CxFV(prM-E), respectively

Table 2. Primers used during the construction of full-length flavivirus chimeric DNAs

Primer	Polarity	Sequence[a]	Target
M-F1	Sense	5’ACATTTCCCCGAAAAGTGCCACCTGACGTCTCGAC3’	Cloning vector
MW-R1	Antisense	5’CCTCCTGGTTTCTTAGACATTCCCGCCACAAAAAGTGG3’	WNV/MODV
MWi-R1	Antisense	5’TAACTGCTCCTACGCTGGCGATTGACAATATGGTTCCCATCATCC3’	WNV/MODV
MC-R1	Antisense	5’CTTACCGTCGTCCTTTCCCATTCCCGCCACAAAAAGTGG3’	CxFV/MODV
MCi-R1	Antisense	5’ACGGCGCCCAGCACCATCATTGACAATATGGTTCCCATCATC3’	CxFV/MODV
MW-F2	Sense	5’CCACTTTTTGTGGCGGGAATGTCTAAGAAACCAGGAGG3’	MODV/WNV
MWi-F2	Sense	5’ATGGATGATGGGAACCATATTGTCAATCGCCAGCGTAGGAGCAG3’	MODV/WNV
MC-F2	Sense	5’CCACTTTTTGTGGCGGGAATGGGAAAGGACGACGGTAAG3’	MODV/CxFV
MCi-F2	Sense	5’ATATGGATGATGGGAACCATATTGTCAATGATGGTGCTGGGCGCCGTC3’	MODV/CxFV
MW-R2	Antisense	5’CAAGGACACAGCCATGATCAGCGTGCACGTTCACGGAG3’	MODV/WNV
MC-R2	Antisense	5’CATCAAGGACACAGCCATGATCTGCCTTGGTGTAGATAAAGTATCC3’	MODV/CxFV
MW-F3	Sense	5’CTCCGTGAACGTGCACGCTGATCATGGCTGTGTCCTTG3’	WNV/MODV
MC-F3	Sense	5’GGATACTTTATCTACACCAAGGCAGATCATGGCTGTGTCCTTGATG3’	CxFV/MODV
M-R3	Antisense	5’TCCATTTGCATTGATGACTGGAGAACCAGATGAACCAGGAGG3’	MODV
M-F4	Sense	5'-AGACTCTTATTCTTGGGGTGGG-3'	MODV
T7MOD-F	Sense	5’**TAATACGACTCACTATAGG**AGTTGATCCTGCCAGCGGTG3’	**T7**/MODV
M-R10600	Antisense	5’AGCGGAGGTCATATTCATGACCACACAGATTACATG3’	MODV

[a]Heterologous virus sequences are underlined in chimeric primers, T7 promoter sequence is bolded

Table 3. Ability of MODV-WNV(prM-E) to induce CPE in vertebrate and mosquito cell cultures

Passage History	[1]CPE	[2]RT-PCR
Vero	-	+
Vero + Vero	-	+
Vero + Vero + Vero	+	+
BHK-21	[3]ND	+
BHK-21 + Vero	+	+
BHK-21 + Vero + Vero	+	+
BHK-21 + Vero + Vero + C6/36 + C6/36 + C6/36	-	-
C6/36	-	-
C6/36 + C6/36	-	-
C6/36 + C6/36 + C6/36	-	-

[1]Cells were monitored regularly for up to 4 days (BHK-21 cells) or 7 to 9 days (Vero and C6/36 cells) for the presence (+) or absence (-) of CPE

[2]Cell culture supernatants were assayed by RT-PCR for the presence (+) or absence (-) of MODV-WNV(prM-E)-specific sequences. RNA samples were treated with DNase I prior to RT-PCR amplification

[3]Not determined due to the presence of extensive cell death in the negative control cultures

Table 4. Mutations accrued in the C-prM-E genes of MODV-WNV(prM-E) during transfection and passage in designated cell types

Passage History	Nucleotide Position	Amino Acid Position	Nucleotide Change	Amino Acid Change
Original Inoculum (BSR-T7)	1457	E-167	C → T	Leu → Phe
BHK-21 + Vero + Vero	323	C-72	T → C	Silent
	1457	E-167	C → T	Leu → Phe
	1771	E-271	T → C	Silent
	2372	E-472	A → G	Met → Val
Vero + Vero + Vero	462	prM-2	C → T	Thr → Ile
	1307	E-117	G → A	Ala → Thr
	1457	E-167	C → T	Leu → Phe
	1894	E-216	T → C	Silent
	2261	E-435	T → C	Phe → Leu

CHAPTER 5

GENERAL CONCLUSIONS

While arthropod-borne flaviviruses continuously cause severe hemorrhagic fever and encephalitis in humans and animals and are the subject of extensive research, several insect-specific flaviviruses (ISFs) have been discovered and isolated from various mosquito species around the world. The attention on ISFs has increased due to their possible impact on the transmission of arthropod-borne viruses in co-infected vectors. Flaviviruses with no known arthropod vector (NKV), another single-host flavivirus, have also received more attention recently because of their potential to be models for encephalitic flavivirus studies. Moreover, data obtained from phylogenetic analyses of ISFs and NKVs have provided more information on flavivirus evolution and may provide insight on emerging and re-emerging flavivirus diseases; thus, future research on these two groups of flaviviruses is clearly warranted.

Although numerous ISFs have been discovered and are widespread in nature, little is known on their transmission dynamics and tissue tropisms as well as their interaction with arthropod-borne flaviviruses in nature. Evidence of vertical transmission in various ISFs has been reported based on their detection in field-collected larvae, pupae and adult male mosquitoes as well as the evidence provided in experimentally infected mosquitoes (Bolling et al., 2011; Bolling et al., 2012; Cook et al., 2006; Farfan-Ale et al., 2010; Lutomiah et al., 2007; Sang et al., 2003). The data in chapter 2 supports and extends these observations as we provide evidence of efficient transovarial

transmission (TOT) by CxFV in *Culex pipiens* mosquitoes in nature. The filial infection rate (FI) and TOT rates reported in this study are dramatically higher than the less than 1% FI and vertical transmission rates typically reported in mosquito-borne flaviviruses (Aitken et al., 1979; Beaty et al., 1980; Francy et al., 1981; Hardy et al., 1984; Kay and Carley, 1980; Rosen et al., 1978; Tesh, 1980). Moreover, the tissue tropism experiments revealed that CxFV establishes a systemic infection in mosquito hosts as CxFV RNA was detected in all of the mosquito organs examined including the ovaries. In contrast, mosquito-borne flaviviruses rarely disseminate to the ovaries of infected mosquitoes, consistent with the inefficient vertical transmission rate reported for these dual-host viruses (Girard et al., 2004; Turell, 1988; Zhang et al., 2010). These differential findings between mosquito-borne flaviviruses and ISFs provide fundamental knowledge for further in-depth studies into virus-host interactions particularly the processes that occur in the mosquito ovary during mosquito-borne virus infection. Further studies on other modes of transmission of ISFs are also warranted in order to better understand how these viruses are maintained in nature.

From our study, it is interesting that CxFV RNA was detected in the salivary glands of infected mosquitoes because, as a result of the inability of this virus to infect vertebrates, establishment of a salivary gland infection does not appear necessary for ISFs to persist in nature. These findings imply that the viral genetic determinants needed for vertebrate-mosquito flaviviruses to disseminate to the salivary glands of their mosquito vectors have been maintained by viruses in the insect-specific lineage. This implication is correlated to the theory that ISFs may have evolved from mosquito-borne

flavivirus or that they have evolved together and then the ISFs lost their ability to replicate in vertebrate hosts (Gould et al., 2003; Kuno et al., 1998). More studies are needed to better understand flavivirus evolution. Recently, Kent et al. (2010) demonstrated that CxFV is not secreted into the saliva of infected *Cx. quinquefasciatus.* These data, together with our findings, could indicate that CxFV disseminates to, but replicates poorly in, the salivary glands of infected *Culex* spp. mosquitoes, thereby resulting in viral titers that do not support efficient secretion into the saliva. Another explanation is that *Culex* spp. mosquitoes possess a salivary escape barrier that inhibits the secretion of CxFV into the saliva. Interestingly, however, CxFV was present in the saliva of mosquitoes co-infected with CxFV and WNV (Kent et al., 2010) which implies that, under certain conditions, the potential salivary escape barrier can be overcome. More investigations are needed to elucidate the mechanisms of virus dissemination to the salivary glands and secretion into the saliva of mosquitoes co-infected with insect-specific and arthropod-borne flaviviruses. At present, the impact that ISFs have on transmissibility of pathogenic flaviviruses by arthropod vector is unclear. There are only a few studies published to date on the vector competence of mosquitoes co-infected with ISFs and pathogenic flaviviruses, and both negative and positive effects by enhancing and interfering with the transmissibility of pathogenic flaviviruses have been observed. Due to the limited data to date and the variations of results that have been reported, further studies are still needed to clarify interactions between ISFs and arthropod-borne flaviviruses in arthropod hosts in nature. Nevertheless, according to the broad range of genetic diversity within ISFs and within arthropod-borne flaviviruses, variable outcomes

may be observed due to the different species of viruses and mosquito used in these studies.

Previously, Farfan-Ale et al. (2009) reported a high prevalence of CxFV in *Cx. quinquefasciatus* in the Yucatan Peninsula of Mexico. The prototype Mexican strain of CxFV (designated CxFV-Mex07), which was isolated from *Cx. quinquefasciatus* in the Yucatan Peninsula of Mexico in 2007, represents the only CxFV isolate from Mexico for which genomic sequence data are available (Farfan-Ale et al., 2009). Furthermore, *Cx. quinquefasciatus* is the only mosquito species from which the Mexican strain of CxFV has been isolated (Farfan-Ale et al., 2009; Farfan-Ale et al., 2010). In chapter 3, we demonstrate that the host-range of CxFV in the Yucatan Peninsula of Mexico is not restricted to *Cx. quinquefasciatus* and provide evidence of limited genetic and phylogenetic diversity between CxFV isolates in this region. Further investigations on sequence analyses of additional ISFs are essential in providing more information for better understanding of evolutionary relationships among ISFs and other flaviviruses.

In chapter 4, we report the first chimeric flavivirus to be constructed using a NKV flavivirus as the backbone. The chimeric virus, which contains the prM-E genes of WNV, could replicate within vertebrate but not mosquito cells indicating that genetic elements outside of the prM-E gene region of MODV condition its vertebrate-specific phenotype. This study also reports the first attempts to create a chimeric flavivirus between an ISF and NKV flavivirus. Two constructs were generated; one containing all of the structural genes of CxFV and the other containing the CxFV prM-E genes in a MODV backbone, but neither yield detectable virus. Most success in the generation of

chimeric flaviviruses has been achieved through prM-E gene substitutions. However, unlike our study, all previous studies were performed using flaviviruses that share a common host. These findings indicate that the successful generation of chimeric viruses between ISFs and NKV flaviviruses will prove extremely challenging due to the evolutionary divergence and differential host ranges of these viruses. The genus *Flavivirus* consists of three distinct groups that have different host specificities: arthropod/vertebrate, vertebrate-specific, and insect-specific. Thus far, the genetic elements that determine host range of flavivirus have not yet been identified. Identification of genetic determinants that have conditioned insect-specific and vertebrate-specific host ranges of ISFs and NKVs respectively will provide insight into the mechanisms that allow arthropod-borne viruses to cycle between vertebrates and arthropod vectors. This knowledge will provide more useful information for rational vaccine design or creating mechanisms to control vector populations.

In summary, it is clear that further studies are important for a better understanding of the complicated and vastly different genetic and host range diversity of flaviviruses and the first priority is to find efficient treatments or effective control and prevention strategies for diseases caused by arthropod-borne flaviviruses. Comparative studies between single- and dual-host members of the *Flavivirus* genus will help us understand why some flaviviruses can infect only vertebrate or only invertebrate organisms while other flaviviruses can infect both insect and vertebrate hosts and cause devastating disease in humans and animals. These future studies will provide us with more knowledge on viral evolution, host specificity, and viral transmissibility and may

also provide insight on emerging and re-emerging diseases as well as useful information for creating efficient disease control and prevention strategies such as vaccine development.

References

Aitken, T.H., Tesh, R.B., Beaty, B.J., Rosen, L., 1979, Transovarial transmission of yellow fever virus by mosquitoes (*Aedes aegypti*). Am J Trop Med Hyg 28, 119-121.

Beaty, B.J., Tesh, R.B., Aitken, T.H., 1980, Transovarial transmission of yellow fever virus in *Stegomyia* mosquitoes. Am J Trop Med Hyg 29, 125-132.

Bolling, B.G., Eisen, L., Moore, C.G., Blair, C.D., 2011, Insect-Specific Flaviviruses from *Culex* Mosquitoes in Colorado, with Evidence of Vertical Transmission. The American Journal of Tropical Medicine and Hygiene 85, 169-177.

Bolling, B.G., Olea-Popelka, F.J., Eisen, L., Moore, C.G., Blair, C.D., 2012, Transmission dynamics of an insect-specific flavivirus in a naturally infected *Culex pipiens* laboratory colony and effects of co-infection on vector competence for West Nile virus. Virology 427, 90-97.

Cook, S., Bennett, S.N., Holmes, E.C., De Chesse, R., Moureau, G., de Lamballerie, X., 2006, Isolation of a new strain of the flavivirus cell fusing agent virus in a natural mosquito population from Puerto Rico. J Gen Virol 87, 735-748.

Farfan-Ale, J.A., Lorono-Pino, M.A., Garcia-Rejon, J.E., Hovav, E., Powers, A.M., Lin, M., Dorman, K.S., Platt, K.B., Bartholomay, L.C., Soto, V., Beaty, B.J., Lanciotti, R.S., Blitvich, B.J., 2009, Detection of RNA from a Novel West Nile-like virus and high prevalence of an insect-specific flavivirus in mosquitoes in the Yucatan Peninsula of Mexico. Am J Trop Med Hyg 80, 85-95.

Farfan-Ale, J.A., Lorono-Pino, M.A., Garcia-Rejon, J.E., Soto, V., Lin, M., Staley, M., Dorman, K.S., Bartholomay, L.C., Hovav, E., Blitvich, B.J., 2010, Detection of flaviviruses and orthobunyaviruses in mosquitoes in the Yucatan Peninsula of Mexico in 2008. Vector Borne Zoonotic Dis. Apr 6.

Francy, D.B., Rush, W.A., Montoya, M., Inglish, D.S., Bolin, R.A., 1981, Transovarial transmission of St. Louis encephalitis virus by *Culex pipiens* complex mosquitoes. Am J Trop Med Hyg 30, 699-705.

Girard, Y.A., Klingler, K.A., Higgs, S., 2004, West Nile virus dissemination and tissue tropisms in orally infected *Culex pipiens quinquefasciatus.* Vector Borne Zoonotic Dis 4, 109-122.

Gould, E.A., de Lamballerie, X., Zanotto, P.M.d.A., Holmes, E.C., 2003, Origins, evolution, and vector□host coadaptations within the Genus Flavivirus, In: Advances in Virus Research. Academic Press, pp. 277-314.

Hardy, J.L., Rosen, L., Reeves, W.C., Scrivani, R.P., Presser, S.B., 1984, Experimental Transovarial Transmission of St. Louis Encephalitis Virus by *Culex* and *Aedes* Mosquitoes. Am J Trop Med Hyg 33, 166-175.

Kay, B.H., Carley, J.G., 1980, Transovarial transmission of Murray Valley encephalitis virus by *Aedes aegypti* (L). Aust J Exp Biol Med Sci 58, 501-504.

Kent, R.J., Crabtree, M.B., Miller, B.R., 2010, Transmission of West Nile Virus by *Culex quinquefasciatus* Say Infected with Culex Flavivirus Izabal. PLoS Negl Trop Dis 4, e671.

Kuno, G., Chang, G.J., Tsuchiya, K.R., Karabatsos, N., Cropp, C.B., 1998, Phylogeny of the genus Flavivirus. J Virol 72, 73-83.

Lutomiah, J.J.L., Mwandawiro, C., Magambo, J., Sang, R.C., 2007, Infection and Vertical Transmission of Kamiti River Virus in Laboratory Bred *Aedes aegypti* Mosquitoes. Journal of Insect Science 7, 1-7.

Rosen, L., Tesh, R.B., Lien, J.C., Cross, J.H., 1978, Transovarial transmission of Japanese encephalitis virus by mosquitoes. Science 199, 909-911.

Sang, R., Gichogo, A., Gachoya, J., Dunster, M., Ofula, V., Hunt, A., Crabtree, M., Miller, B., Dunster, M., 2003, Isolation of a new flavivirus related to Cell-fusing agent virus (CFAV) from field collected flood water *Aedes* mosquitoes sampled from a Dambo in central Kenya. Arch Virol 148, 1085 - 1093.

Tesh, R.B., 1980, Experimental studies on the transovarial transmission of Kunjin and San Angelo viruses in mosquitoes. Am J Trop Med Hyg 29, 657-666.

Turell, M.J., 1988, Horizontal and vertical transmission of viruses by insect and tick vectors, In: Monath, T.P. (Ed.) The Arboviruses: Epidemiology and Ecology. CRC Press, Boca Raton, FL., pp. 127-152.

Zhang, M., Zheng, X., Wu, Y., Gan, M., He, A., Li, Z., Liu, J., Zhan, X., 2010, Quantitative Analysis of Replication and Tropisms of Dengue Virus Type 2 in *Aedes albopictus*. The American Journal of Tropical Medicine and Hygiene 83, 700-707.

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